Localization of Chitin Synthase in *Mucor rouxii* by an Autoradiographic Method

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The localization of chitin synthase in the cells of *Mucor rouxii* was studied by a method which combined permeabilization of the cells with toluene/ethanol and incubation with the radioactive substrate UDP-[^{3}H]GlcNAc followed by high resolution autoradiography. By this technique it was demonstrated that most of the chitin synthesized by these cells was located within the cytoplasm, and only a small amount of the enzyme product appeared at the cell surface. It was concluded that most of the chitin synthase of *M. rouxii* is located in the cytoplasm of the cells.

INTRODUCTION

Chitin, a linear (1→4)-β-*N*-acetylglucosaminyl polymer, is a major fibrillar component of most fungal cells, where it is responsible for the shape and rigidity of their cell walls. Synthesis of chitin has been carried out *in vitro* by subcellular preparations obtained from different species of fungi (for a review, see Ruiz-Herrera, 1982). One aspect of chitin biosynthesis that has remained controversial is the subcellular distribution of the enzyme system involved. It has been reported that almost all the enzyme activity in protoplasts from *Saccharomyces* (Duran *et al.*, 1975) and *Candida albicans* (Braun & Calderone, 1978) was bound to the plasma membrane, and Vermeulen *et al.* (1979) reported that in protoplasts from *Schizophyllum commune* 50% of the enzyme activity was associated with the plasma membrane and about 30% was sedimented as a system of particles by high-speed centrifugation. In contrast, Bracker *et al.* (1976), Ruiz-Herrera *et al.* (1977) and Bartnicki-Garcia *et al.* (1978) isolated most of the chitin synthase from different fungi in the form of cytoplasmic microvesicular organelles (chitosomes) and demonstrated that these organelles were capable of forming chitin microfibrils *in vitro*. Previous work (Sentandreu & Ruiz-Herrera, 1978; Dominguez *et al.*, 1980; Flores-Carreon *et al.*, 1980) demonstrated that chitin synthase could be detected *in situ* by permeabilization of fungal cells with small amounts of toluene/ethanol, a method originally introduced for the assay of cytoplasmic enzymes in whole cells (Reeves & Sols, 1973; Serrano *et al.*, 1973). In the present work we employed this technique of cell permeabilization in combination with high-resolution autoradiography to study the cellular localization of chitin synthase in *Mucor rouxii*. We present evidence that supports a cytoplasmic location of the enzyme.

METHODS

Organism and culture conditions. Mucor rouxii IM-80 (ATCC 24905) was used in this study. Spores (2×10^8) were inoculated into 200 ml of liquid complex (YPG) medium (Bartnicki-Garcia & Nickerson, 1962) and incubated with shaking at 28 °C. After about 6 h, germlings were collected by low-speed centrifugation, washed with chilled 0·05 M-KH_2PO_4/NaOH (phosphate) buffer pH 6·5 containing 10 mM-$MgCl_2$ and finally resuspended in 10 ml of the same buffer.

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Cell permeabilization. The cells were mixed with an ethanol/toluene mixture (50%, v/v) to give a final concentration of 1% (v/v). This suspension was subjected to Vortex mixing for ten 30 s periods, with 2 min intervals of cooling in an ice bath. The cells were then washed five times with cold buffer and finally resuspended in 4 ml of buffer.

Chitin synthase assays. The chitin synthase activity of permeabilized cells was measured essentially as previously described (Sentandreu & Ruiz-Herrera, 1978; Flores-Carreon *et al.*, 1980). Reaction mixtures contained: 0·5 mM-uridine 5′-diphosphate *N*-acetyl-D-[^{14}C]glucosamine (UDP-[^{14}C]GlcNAc) (sp. act. 0·163 Ci mol^{-1}; 6·03 GBq mol^{-1}), 0·2 mM-ATP, 20 mM-*N*-acetylglucosamine (GlcNAc), 10 mM-$MgCl_2$, 0·5 M-phosphate buffer pH 6·5 and permeabilized cells in a final volume of 125 µl. Chitin synthase was normally activated by the addition of either trypsin (1 mg ml^{-1}) or rennilase (3 mg ml^{-1}). Samples were incubated at 22 °C for various periods. The reaction was stopped by the addition of either 2–3 drops of glacial acetic acid or 0·5 ml 2% (v/v) glutaraldehyde in 0·1 M-cacodylate buffer pH 7·1. Samples were filtered through a fibreglass filter (Schleicher & Schull no. 8, Dassel, FRG) and washed five times with 20 ml each of 1 M-acetic acid/ethanol (70:30, v/v), or, after overnight incubation, with 5 ml 2% glutaraldehyde followed by five washings with 20 ml each of 0·5 M-Tris buffer pH 7·0 containing 10 mM-$MgCl_2$. The filters were dried and radioactivity was counted with a scintillation spectrometer.

Preparation of samples for autoradiography. Samples (2 ml) of toluenized cells were incubated at room temperature for 60 min in reaction mixtures containing: 0·05 M-phosphate buffer pH 6·5, 10 mM-$MgCl_2$, 40 mM-GlcNAc and 2 mM-UDP[^{3}H]GlcNAc [10 Ci mol^{-1} (370 GBq mol^{-1}); 20 µCi ml^{-1} (740 kBq ml^{-1})]. One sample received 1 mg trypsin ml^{-1} to activate the chitin synthase zymogen. Incorporation was stopped by the addition of 20 ml 2% glutaraldehyde in 0·1 M-cacodylate buffer pH 7·1 and samples were treated as described below.

Electron microscopy. Cells were fixed overnight in 2% glutaraldehyde in 0·1 M-cacodylate buffer pH 7·1 at 0°C, washed four times with 50 mM-Tris buffer pH 7·0 containing 10 mM-$MgCl_2$, and embedded in Araldite resin. The technique used for autoradiography has been previously described (Martinez-Ramon *et al.*, 1975). Thin sections (80 nm) were collected over celloidine film (2%, w/v, in isoamyl acetate). The dry sections were stained with 5% (w/v) uranyl acetate in water for 1 h and finally coated with a carbon film about 10 nm thick. The nuclear emulsion used was Ilford L4 diluted to 1/4 strength (v/v), and the exposure time was 4 weeks at 4°C. The silver grains were developed with ascorbic acid/fenidon after previous treatment for 30 sec with a solution containing (ml^{-1}): 0·04 mg $AuCl_3$, 0·5 mg KSCN and 0·5 mg KBr. Finally the sections were contrasted with lead citrate and examined and photographed with a Philips 300 electron microscope.

Quantitative autoradiography. Photographs of the cell cross-sections were divided into three domains: (1) external, an area 500 nm wide immediately external to the cell wall; (2) cortical, a similar area from the external limit of the wall toward the interior of the cell, i.e. embracing the cell envelope; and (3) the remainder of cytoplasm of the cell. The numbers of silver grains in each domain were recorded for 30 cell cross-sections, and the total cross-sectional area was determined by a photogravimetric method. Results were expressed as total and specific activity. Specific activity was expressed as the number of silver grains per 100 µm^2. The significance of the results was analysed by the method of Whur *et al.* (1969), where the percentage of total silver grains is calculated for each domain. This value corresponds to the 'uncorrected grain distribution'. The method eliminates non-significant grains by subtracting the percentage value of the surface corresponding to each domain from the percentage of grains found in the equivalent domain. These calculated values, designated 'corrected grain distribution', are considered significant only if they show positive values.

Miscellaneous. UDP-GlcNAc was from Sigma; UDP-[^{14}C]GlcNAc was from Amersham. Polyoxin D was obtained from S. Bartnicki-Garcia, University of California, Riverside, USA, through the courtesy of M. Sakamaki, Kaken Chemical Co. (Tokyo, Japan). A mixture of nikkomycins X and Z was a generous gift from H. Zahner, University of Tübingen, FRG. Rennilase, an acid protease from *Mucor miehei*, was a gift from Novo Enzyme Corporation (Mamaroneck, NY, USA).

RESULTS

As described previously (Sentandreu & Ruiz-Herrera, 1978), intact germlings of *M. rouxii* incubated with labelled UDP-GlcNAc did not synthesize chitin in significant amounts, whereas those treated with toluene incorporated the label in amounts that increased in proportion to the incubation time. The addition of an activating protease increased chitin synthesis, but contrary to results obtained with cell-free extracts or purified chitosomes (Ruiz-Herrera & Bartnicki-Garcia, 1976; Ruiz-Herrera *et al.*, 1977), trypsin was more effective as an activator than rennilase (Fig. 1). Probably the larger molecular weight of the latter impairs its penetration through the cell wall. Polyoxin and nikkomycin almost completely inhibited GlcNAc incorporation by permeabilized cells (Fig. 1). The lower inhibitory effect of polyoxin was possibly due to the fact that the sample used had been kept for several years in the freezer and may have lost some of its potency.

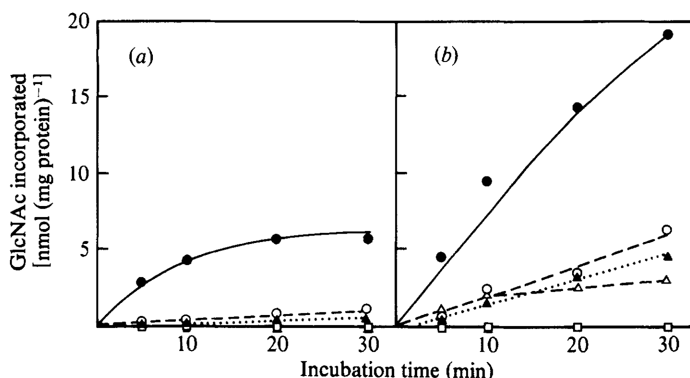

Fig. 1. Chitin biosynthesis by permeabilized cells of *Mucor rouxii*. Effect of protease treatment and addition of inhibitors. Germinating spores treated with toluene/ethanol were incubated with substrate and additions as described in Methods. At intervals, samples were removed, the reaction was stopped, the cells were filtered, washed and dried, and radioactivity incorporated was measured. (*a*) Cells incubated with rennilase. (*b*) Cells incubated with trypsin. ●, No inhibitor added; ○, plus 20 μM-polyoxin; ▲, plus 5 μM-nikkomycin; □, plus 20 μM-nikkomycin; △, no protease added.

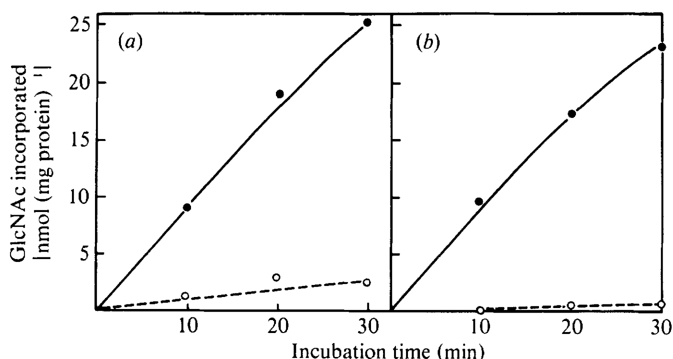

Fig. 2. Efficiency of washing for the removal of soluble radioactive compounds from permeabilized cells. Germinating spores were treated and incubated as described for Fig. 1. (*a*) The reaction was stopped with glacial acetic acid and the cells washed with acetic acid/ethanol. (*b*) The reaction was stopped with glutaraldehyde and the cells washed with glutaraldehyde and Tris buffer. ●, No inhibitor added; ○, plus 5 μM-nikkomycin.

Incubated cells washed with either acetic acid/ethanol or Tris buffer after fixation with glutaraldehyde (see Methods) gave similar results for radiolabel incorporation (Fig. 2). No significant incorporation was observed in either case when nikkomycin was present in the incubation media. These results show that all free substrate and soluble by-products were effectively extracted from the permeabilized cells during washings, with only chitin remaining in the washed cells.

Toluene-treated germinating spores incubated for 60 min with UDP-[^{3}H]GlcNAc under the conditions described in Methods were fixed and prepared for autoradiography. Figs 3 and 4 are representative electron micrographs of these cells. Germ tubes are recognizable by their wall being thinner than those of the spores. With regard to silver grain distribution, several observations may be pointed out: firstly, extracellular background is very low, only a few grains appearing distant from the cells; secondly, most grains appear in the interior of the cells; thirdly, grain distribution is not homogeneous, since grains appear distinctly clustered; and finally, grains are not preferentially located in the growing region of the germlings but appear randomly distributed in the cells. No significant difference in the grain distribution was observed between the cells incubated in the presence of trypsin and those incubated in its absence, although grains in the former were more abundant (see below).

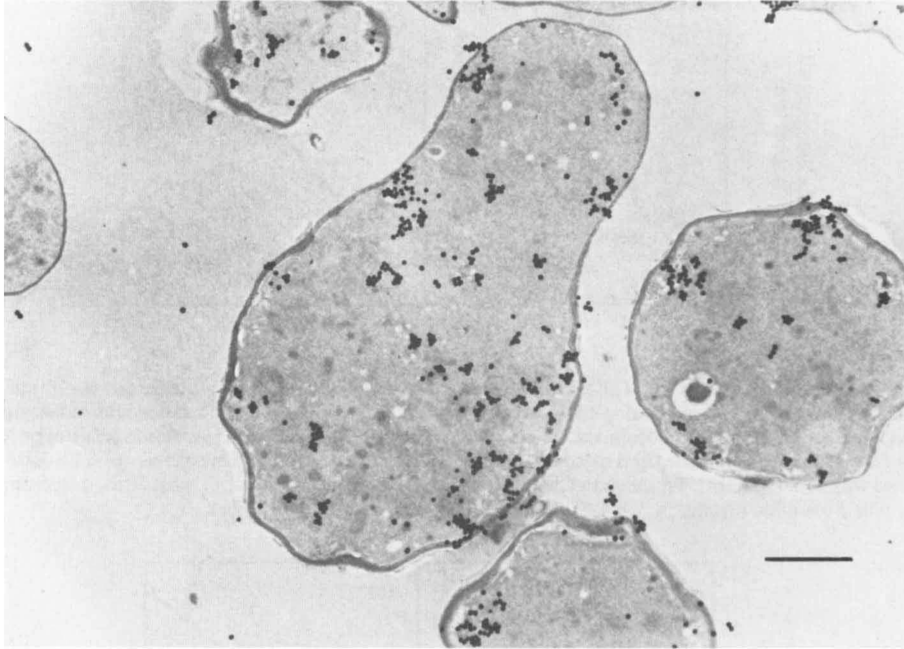

Fig. 3. Electron micrograph showing a typical field of cells of *M. rouxii* incubated with UDP-[^{3}H]GlcNAc in the presence of trypsin and developed for autoradiography. Notice the low background number of grains and their accumulation in the cytoplasm of the cells. Bar marker, 2 µm.

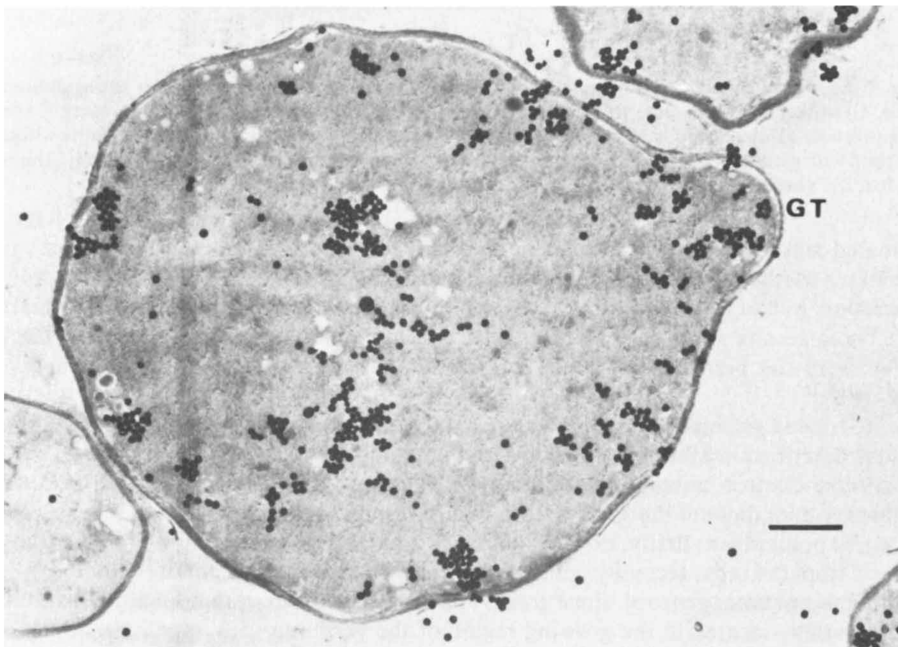

Fig. 4. A cell of *M. rouxii* showing the accumulation of grains in the internal zone of the cell. This cell was incubated with labelled substrate in the presence of trypsin. GT, germ tube; notice the thinner cell wall. Bar marker, 1 µm.

Table 1. *Silver grain distribution within cross-sections of Mucor rouxii*

Zone*	Parameter†	Control cells	Trypsin-treated cells
External (500 nm band)	Total activity	217	654
	Area (μm²)	403	448
	Specific activity	53·8	145·9
Cortical (500 nm band)	Total activity	482	2407
	Area (μm²)	397	440
	Specific activity	121·4	547·0
Remainder of cytoplasm	Total	1912	7263
	Area (μm²)	1560	1838
	Specific activity	122·5	392·2

* For description of zones see Methods.
† Total activity is defined as the number of silver grains in 30 cell cross-sections, and specific activity as the number of silver grains per 100 μm².

Table 2. *Analysis of the significance of the distribution of silver grains within cross-sections of Mucor rouxii*

	Uncorrected activity (%)		Surface (%)		Corrected activity†	
Zone*	Control	+ Trypsin	Control	+ Trypsin	Control	+ Trypsin
External	8·31	6·33	17·07	16·43	−8·76	−10·10
Cortical	18·46	23·30	16·82	16·14	1·64	7·16
Remainder of cytoplasm	73·32	70·31	66·10	67·42	7·12	2·89

* For description of zones see Methods.
† Calculated as described in Methods; positive values are considered to be significant.

Quantitative evaluation of the silver grain distribution is shown in Table 1. It may be observed that most of the radioactivity was present in the interior of the cells, whereas the external zone contained less radioactivity. In cells incubated with trypsin, radioactivity increased in the three zones by the following factors: 2·71 for the external zone, 4·50 for the cortical zone and 3·22 for the intracellular zone. Specific activity was similar for the cortical and internal zones, although trypsin-treated cells had a higher specific activity in the cortical zone. The specific activity of the external zone was about one third of the internal radioactivity.

The significance of the silver grain distribution results was analysed as described in Methods. Only the activity found inside the cells was significant; the values found external to the cell wall were not significant (Table 2).

DISCUSSION

Some of the previous techniques used for determination of chitin synthase localization introduce, in our opinion, artefacts that limit the value of the results. In particular, the preparation of protoplasts involves lengthy and aggressive treatment. Therefore profound structural and metabolic alterations may occur as suggested by the extreme lability of apical vesicles to changes in the environment (Girbardt, 1957), and the fact that yeast protoplasts show a lag of 60–90 min before reinitiation of protein synthesis under optimal growth conditions (R. Sentandreu, unpublished observations). On the other hand, mechanical breakage of the cells may destroy the integrity of membranes and other labile structures. For this reason cytoplasmic localization of chitin synthase has been assessed by the use of other techniques for cell breakage, e.g. osmotic shock of the wall-less mutant of *Neurospora crassa* (Bartnicki-Garcia *et al.*, 1980) and removal of cytoplasmic material from the tip of *Phycomyces blakesleeanus* sporangiophores by use of a microsyringe (Herrera-Estrella *et al.*, 1982).

To avoid some of the above-mentioned problems we have used a cell permeabilization method that causes a rapid halt in cell metabolism. Treatment of cells with small amounts of

toluene/ethanol produces an outflow of ions and other small metabolites, but labile enzyme systems such as glucan synthase (Sentandreu *et al.*, 1975), chitin synthase (Sentandreu & Ruiz-Herrera, 1978; Flores-Carreon *et al.*, 1980) in whole cells, and even chitin synthase in isolated chitosomes (S. Bartnicki-Garcia & R. Sentandreu, unpublished observations) remain fully active. These results stress the non-destructive character of the technique used.

Incubation of these permeabilized cells with the sugar-nucleotide substrate for chitin biosynthesis leads to accumulation of the insoluble product in the vicinity of the enzyme, since all the normal delivery mechanisms of the cell are disrupted. Autoradiography of these cells thus permits the localization of chitin synthase in the cell. The removal of residual substrate and soluble by-products from glutaraldehyde-fixed cells by washing with buffer is evidence that the only remaining radioactivity derives from an insoluble product, which is considered to be chitin by two observations: its amount is increased by proteolysis, and its accumulation is almost abolished by the chitin synthase inhibitors polyoxin and nikkomycin.

The results obtained establish that most of the chitin synthesized by permeabilized cells is located in the cytoplasm. According to quantitative autoradiography data from Salpeter *et al.* (1969) we calculated that an area 500 nm wide on each side of the cell surface would register about 80% of the radioactivity originating from a source located in the plasma membrane and/or the cell wall. [The cell wall of the spore is 100–160 nm thick and that of the germ tube 40–60 nm thick (Bartnicki-Garcia *et al.*, 1968)]. Therefore it would be expected that, statistically, grain distribution would be equal at both sides of the cell surface. This was not the case, and in fact the amount of grains located external to the cells was statistically not significant. This means that most of the grains in the cortical zone came from internal sources. A word of caution must be raised however. Although non-significant (negative) values were obtained for the external zone, its specific activity increased after trypsin treatment. This result suggests that some (although very low) activity of chitin synthase may be located at the cell surface. The fact that the specific activities of the cortical and internal zones are about the same suggests that they do not represent significantly different enzyme compartments. Active chitin synthase detected in the cytoplasm of cells incubated without trypsin may represent either enzyme activated during the treatment with toluene or active molecules in transit to their final destination at the cell surface.

In conclusion, the results reported in this paper support and extend previous observations that showed that chitin synthase in *M. rouxii* is localized mostly in the cytoplasm.

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