Formation and Regeneration of *Geotrichum candidum* Protoplasts

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SUMMARY

The formation of protoplasts from *Geotrichum candidum*, by means of a lytic enzyme complex of *Streptomyces satsumaensis*, was considerably stimulated by adding thiols, including dithiothreitol, 2-mercaptoethanol and cysteamine, to the incubation medium. Treatment of the mycelium with these compounds before incubation with lytic enzyme also enhanced protoplast formation, dithiothreitol giving the most rapid release of protoplasts. The same stimulating effect could be obtained with a proteolytic enzyme. These results suggest the presence of proteinaceous material in the outer layers of the hyphal wall. *Geotrichum candidum* protoplasts regenerated in liquid as well as solid media if a suitable osmotic stabilizer was present. Up to 20 % of the protoplasts could grow into new mycelium.

INTRODUCTION

Geotrichum candidum produces an extracellular lipase (Wouters, 1966). To understand the process of secretion of this enzyme Sietsma & Wouters (1971) have studied the composition of the wall because the wall may play an important role in secretion mechanisms (Lampen, 1968). Another way of approaching the problem of enzyme secretion is to study the process in cells without walls (Kuo & Lampen, 1971; Liras & Gascón, 1971). Sietsma & Wouters (1971) have described the preparation of protoplasts from the mycelium of a young (24 h) culture of *G. candidum*. The present study was initiated to get protoplasts from mycelium of older cultures as well, for lipase production starts only after 5 to 7 days of cultivation (Wouters, 1966). A study of the regeneration of protoplasts was also made.

METHODS

Cultures. The organism, *Geotrichum candidum* strain 5, and the culture conditions were as described previously (Sietsma & Wouters, 1971). Culture flasks were incubated either on an orbital shaker or stationarily.

The lytic enzyme complex from *Streptomyces satsumaensis* 1399 was prepared as described by Sietsma & Wouters (1971).

Preparation of protoplasts. In order to get a usable protoplast suspension the arthrospores must be removed from the mycelium. Therefore the culture was filtered over a sieve with a mesh of 32 to 40 μm . The mycelium stayed on this filter whereas the arthrospores were washed through by three to five rinsings with water and one with incubation medium (0.8 M-MgSO₄ in 5 mM-phosphate buffer, pH 5.8). A suspension of mycelium was incubated in 10 ml of this medium with 30 to 50 mg lytic enzyme (freeze-dried preparation) at 30 °C. In some experiments the mycelium was pretreated with thiols, namely 2-mercaptoethanol, cysteamine, thioglycollate and dithiothreitol, or these compounds were added to the incubation medium.

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Protoplasts prepared in this incubation medium sedimented only slowly at 12 000 *g*. Therefore, after incubation, the mycelial debris was separated from the protoplasts by centrifugation at 12 000 *g* for 15 min and washed twice with 0.8 M-MgSO₄. The supernatant fluid and the washings were combined and the density of the liquid decreased by adding two volumes of 0.8 M-NaCl. Afterwards the protoplasts could be sedimented at 4000 *g* for 15 min. Finally they were resuspended in 0.8 M-MgSO₄ or 0.8 M-mannitol in 5 mM-phosphate buffer, pH 5.8.

Regeneration of protoplasts. In regeneration studies the protoplast suspension was supplemented with glucose and glutamate, both in a final concentration of 10 mM, and streptomycin sulphate (250 µg/ml) was added to prevent bacterial growth. The protoplast suspension was then incubated at 30 °C and samples withdrawn at intervals for examination by phase-contrast microscopy. For fluorescence microscopy, sedimented protoplasts were resuspended in a solution of 0.8 M-MgSO₄ and 5 mM-phosphate buffer, pH 5.8, containing 0.1 % Calcofluor White MR (Unilever Research Laboratories, Vlaardingen, The Netherlands) and examined with near u.v. light.

The percentage of protoplasts capable of regenerating was estimated by plating a known number of protoplasts on a solid medium and counting the number of colonies formed after 48 h of incubation at 28 °C. The medium was the *Geotrichum candidum* growth medium supplemented with 0.8 M-mannitol and 1 % agar.

RESULTS AND DISCUSSION

Protoplast formation

To improve the release of protoplasts from yeasts or fungi the use of SH-containing compounds has been frequently recommended (Davies & Elvin, 1964; Bastide, Trave & Bastide, 1971; Sommer & Lewis, 1971). Table 1 shows the effect of the addition of 2-mercaptoethanol, cysteamine, thioglycollate and dithiothreitol on protoplast formation from *Geotrichum candidum* mycelium. Almost total conversion into protoplasts occurred within 2 h when dithiothreitol or 2-mercaptoethanol (final concentration 10 mM) was added to the incubation mixture. Protoplast formation was slightly better from young mycelium than from older, lipase-producing mycelium. 2-Mercaptoethanol, in particular, exhibited a stronger stimulatory effect on protoplast formation from young mycelium. Duell, Inoue & Utter (1964) have shown that pretreatment of stationary-phase cells of *Saccharomyces cerevisiae* with a higher concentration (1.40 M) of 2-mercaptoethanol stimulated protoplast formation considerably and similar treatment made older, lipase-producing mycelium of *G. candidum* as susceptible to the lytic enzymes as younger mycelium.

During protoplast formation, in the absence of sulphydryl-group-containing compounds, swellings appeared at several points on the hyphae, followed by the release of protoplasts (Sietsma & Wouters, 1971). This was also observed with *Aspergillus nidulans* (Peberdy & Gibson, 1971). In the presence of dithiothreitol or 2-mercaptoethanol swelling of hyphae could hardly be observed. The whole mycelial surface seemed to be attacked equally and the bulk of protoplasts emerged simultaneously (Fig. 1*a*). Similar results are mentioned by May (1971), who prepared protoplasts of *Saccharomyces fragilis*.

Pronase added to the incubation medium had the same stimulating effect on protoplast formation as had the thiol compounds (Table 1). This suggests that the hyphal wall of *Geotrichum candidum* is covered with a layer of proteinaceous material which could be removed or loosened by thiol compounds, by their action of reducing S-S linkages in proteins (Nickerson, 1963). Chemical analysis of wall preparations has shown the presence of

Table 1. *Factors affecting formation of Geotrichum candidum protoplasts*

Protoplast formation was followed by phase-contrast microscopy during incubation with lytic enzyme in 0.8 M-MgSO₄ in 5 mM-phosphate buffer, pH 5.8. Pretreatment was performed in the same medium during 30 min at 30 °C. Young mycelium was from a 24 h-shaken culture, old mycelium from a 5-day-stationary culture.

Additions during		Concentration (mM)	Protoplast formation from*	
Incubation	Pretreatment		Young mycelium	Old mycelium
—	—		+	—
Cysteamine	—	500	++	+
Cysteamine	—	100	++	+
Cysteamine	—	10	++	+
—	Cysteamine	500	++	++
—	Cysteamine	50	++	++
2-Mercaptoethanol	—	500	N.T.	●
2-Mercaptoethanol	—	100	++	+
2-Mercaptoethanol	—	10	+++	+
—	2-Mercaptoethanol	500	+++	++
—	2-Mercaptoethanol	170	+++	+++
Dithiothreitol	—	10	+++	+++
Thioglycollate	—	50	+	N.T.
Thioglycollate	—	10	+	N.T.
Pronase	—	2 mg/ml	N.T.	+++

* — = after 10 h no protoplasts; + = after 10 h few protoplasts; ++ = after 6 h many protoplasts, no mycelium left; +++ = after 2 h many protoplasts, no mycelium left; N.T. = not tested.

about 7 % protein (Sietsma & Wouters, 1971). The susceptibility of the wall to lytic enzymes might depend on the nature of this protein layer, which may vary with age of culture and culture conditions.

Regeneration of protoplasts

Protoplast regeneration was generally performed in media stabilized by mannitol, since media stabilized with MgSO₄ gave a lower level of regeneration, as observed for *Pythium* (Sietsma & de Boer, 1973).

Regeneration of protoplasts in liquid medium occurred in a series of well-defined stages (Fig. 1). The first visible signs of the regeneration process could be seen after 2 h. Often protoplasts gave rise to a chain of irregular cells, which were still osmotically fragile (Fig. 1*b*). After 4 h of incubation protoplasts produced a hypha directly or through a chain of yeast-like cells (Fig. 1*c*). At this stage the protoplasts attached to the hyphae were osmotically stable and dilution with distilled water did not cause lysis (Fig. 1*d, e*).

Fluorescence microscopy showed that freshly prepared protoplasts from a 5-day-old culture were not stained with Calcofluor White MR, suggesting that the protoplasts were essentially free of hyphal wall glucans as Calcofluor is known to react principally with glucans (Preece, 1971). After 2 h of regeneration some fluorescent spots appeared on the protoplasts (Fig. 1*f*). The terminal cell of a chain which grew out into a hypha (Fig. 1*g*) and the tip of a growing hypha generally fluoresced most strongly. In an outgrown hypha the fluorescence was concentrated in distinct spots (Fig. 1*h*), which might indicate that glucans are present only at distinct points on the surface or that this polymer layer is partly covered with a Calcofluor-insensitive outer layer (e.g. protein).

Less than 20 % of protoplasts were able to regenerate into new mycelium on the agar medium. Protoplasts prepared in the presence of dithiothreitol from a 24-h-shaken culture

Fig. 1. Formation and regeneration of *Geotrichum candidum* protoplasts. Scale markers represent 10 μm. (a) Protoplasts formed after 2 h of incubation with lytic enzyme in presence of 10 mM-dithiothreitol. (b) Irregularly formed cells after 2 h of regeneration. (c) Outgrowth into a hypha after 4 h of regeneration. (d) Outgrowing cells with hypha. (e) The same as (d) after diluting with water. (f) Regenerating protoplast after 2 h stained with Calcofluor White MR and photographed through a fluorescence microscope. (g) After 4 h (h) After 12 h.

were not able to regenerate, but those from a 5-day-old, lipase-producing culture regenerated. The combined action of lytic enzyme complex and dithiothreitol on young mycelium could produce a protoplast on which no hyphal-wall remnants remain. These walls would then lack any 'primer' necessary for regeneration of wall polymers, as has been suggested by Sommer & Lewis (1971), who prepared protoplasts of *Saccharomyces carlsbergensis* in the presence of dithiothreitol.

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