Formation and Regeneration of Protoplasts from *Conidiobolus lamprauges*

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Protoplasts were formed from aged germlings and mycelia of *Conidiobolus lamprauges*, using a combination of chitinase and β -glucanase. Methanol, ethanol and propanol stimulated protoplast formation, apparently by a mechanism different from that of mercaptoethanol. Regeneration of protoplasts in soft agar was detectable by light microscopy 2 h after incubation and colonies were observable by the naked eye within 40 h; the regeneration rate was 65%.

INTRODUCTION

Chitin is widely distributed in invertebrates, fungi and yeasts but is not found in vertebrates (Muzzarelli, 1977). To date, chitin synthesis by 'chitosomes' (Glaser & Brown, 1957; Ruiz-Herrera *et al.*, 1977; Bartnicki-Garcia *et al.*, 1979) has been investigated. Chitin synthase (EC 2.4.1.16) has not yet been obtained in a highly purified state and its detailed enzymic properties remain unclear. Lipid-linked reactions may be involved. Yeasts possess dolichyl-bound saccharides as intermediates in glycoprotein synthesis (Bretthauer & Wu, 1975), and involvement of lipid-linked reactions in the initiation of chitin synthesis in crustacea was suggested by Horst (1983). Katoh *et al.* (1976) found that conidia of *Penicillium citrinum* could not germinate but grew abnormally into multinuclear giant cells in the presence of sublethal concentrations of tunicamycin, an antibiotic that inhibits the biosynthesis of chitin in insects (Quesada-Allue, 1982).

We have selected the fungus *Conidiobolus lamprauges* for the study of chitin synthesis. It has the following desirable characteristics: (i) it contains chitin in the cell wall; (ii) sufficient growth (and synthesis of chitin) occurs in an appropriate medium; (iii) the cell walls can be removed easily to form protoplasts; and (iv) vegetative cells can be regenerated from protoplasts at a high frequency and a high rate. In this paper we describe the formation and regeneration of protoplasts from *C. lamprauges*.

METHODS

Organism. *Conidiobolus lamprauges* Drechsler no. 454 (= ATCC 46830), isolated from soil (Ishikawa *et al.*, 1979) and maintained in our laboratory, was used throughout the present work.

Culture conditions. The organism was grown at 27 °C on solidified ME medium held in place on the cover of large Petri dishes by a small frame (Fig. 1). ME medium consists of 2% (w/v) Bacto Malt Extract (Difco), 0.1% (w/v) Polypepton (Daigo Eiyokagaku Co., Osaka, Japan), 2% (w/v) glucose and 2% (w/v) agar (Nakarai Chemicals, Kyoto, Japan) in deionized water. When conidia were fully matured, they dropped from the mycelial mats and fell into the 0.05% (w/v) 2-deoxyglucose solution in the dishes. This solution inhibited germination and further growth of the conidia, and kept them at a uniform phase of growth. The conidia thus obtained were washed three times with 0.05 M-sodium phosphate buffer (PB, pH 7.0), and cultured at 27 °C in MY medium, which is composed of 0.3% (w/v) Bacto Malt Extract (Difco), 0.3% (w/v) Bacto Yeast Extract (Difco), 0.5% (w/v) Polypepton (Daigo) and 1% (w/v) lactose in 0.05 M-PB (pH 7.0). At appropriate intervals, mycelia were harvested and examined for their convertibility to protoplasts.

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Fig. 1. Synchronous preparation of *Conidiobolus lamprauges* conidia. (a) Culture dish used for conidia trapping: A, ME medium; B, mat of *C. lamprauges*; C, trapping solution (0.05% 2-deoxyglucose). (b) Conidia trapped in trapping solution. Bar, 25 μ m.

Formation of protoplasts. The standard reaction mixture comprised 20 μ l of an aqueous solution containing 0.4 mg *Arthrobacter luteus* Zymolyase 60000 (Seikagaku Kogyo Co.), 40 μ l *Streptomyces griseus* chitinase solution (Sigma; lyophilized crude powder 0.8 mg), 120 μ l 0.05 M-PB (pH 7.0), 20 μ l 10 M-ethanol or 5 M-2-mercaptoethanol, 200 μ l 2.5 M-D-sorbitol in 0.05 M-PB (pH 7.0), and 600 μ l washed mycelial suspension (1%, v/v, packed mycelium in 0.05 M-PB, pH 7.0). In some experiments, 40 μ l *Helix pomatia* sulphatase solution (Sigma, type H-2) was also added. The reaction mixture was filter-sterilized (Millipore filter, pore size 0.45 μ m) when the protoplasts were to be prepared aseptically. Enzyme digestion was carried out at 30 °C for 2 to 3 h with gentle shaking. After the reaction, the remaining mycelium and cell debris were removed by low-speed centrifugation. The protoplasts formed were washed three times with 0.05 M-D-sorbitol/0.05 M-PB solution (pH 7.0). Protoplast formation was determined by phase-contrast microscopy and by rupture of the protoplasts by dilution. Numbers of protoplasts were counted with a Neubauer-type haematocytometer; the variation was no more than 0.1×10^6 protoplasts ml^{-1} among five experiments.

Regeneration of protoplasts. One hundred protoplasts were aseptically added to 5 ml ME medium containing 0.5% agar and 0.5 M-D-sorbitol, overlaid on ME medium supplemented with 2% (w/v) agar and 0.5 M-D-sorbitol in 9 cm Petri dishes, and incubated at 27 °C. Samples were withdrawn at intervals and regeneration was observed by phase-contrast microscopy.

RESULTS

Enzymes required for protoplast formation

The major polysaccharide components of the cell wall of *Conidiobolus lamprauges* are chitin, β -glucan and an unidentified mannosyl saccharide (Ishikawa *et al.*, 1983). Commercial preparations of chitinase, Zymolyase (β -glucanase) and sulphatase (mostly β -glucuronidase) were examined for their ability to induce protoplast formation (Fig. 2). Individually they did not release protoplasts. Chitinase and sulphatase combined were also ineffective, but Zymolyase plus sulphatase showed a slight activity. When Zymolyase and chitinase were used in combination, almost all the mycelia were converted to protoplasts within 2 h, giving a density of about 2×10^6 protoplasts ml^{-1} . Prolonged incubation resulted in a decrease of protoplast numbers, due to protoplast lysis. Addition of sulphatase to the mixture of Zymolyase and chitinase increased the rate of protoplast formation only slightly.

Enhancers of protoplast formation

Hardly any protoplasts were obtained from *C. lamprauges* by incubation with Zymolyase and chitinase in the absence of mercaptoethanol. Table 1 shows the effect on protoplast formation of several reducing agents, including mercaptoethanol, and of lower alcohols as analogues of mercaptoethanol. Sodium sulphide enhanced protoplast formation, while dithiothreitol and L-ascorbic acid showed weak and no activity, respectively. Lower alcohols were generally as stimulatory as mercaptoethanol. To determine whether mercaptoethanol and lower alcohols acted on the lytic enzymes or on the cells of *C. lamprauges*, these compounds were added to the reaction mixture prior to the addition of the enzymes, incubated at 30 °C for 2 h, and washed off (Table 2). Preincubation of the cells with mercaptoethanol gave no stimulatory effect, whereas ethanol stimulated subsequent cell wall lysis by the enzymes. Preincubation of the cells in

Fig. 2. Effect of lytic enzymes on protoplast formation. Mycelia were treated with Zymolyase plus chitinase plus sulphatase (●), with Zymolyase plus chitinase (○), with Zymolyase plus sulphatase (▲), with chitinase plus sulphatase (△), or without enzymes (□). Ethanol (200 mM) was added to all reaction mixtures. The results are from a typical experiment.

Table 1. *Effect of reducing agents and lower alcohols on protoplast formation*

Addition	Concn (mM)	$10^{-6} \times \text{No. of protoplasts ml}^{-1}$
Deionized water	—	0.12
2-Mercaptoethanol	100	1.51
Dithiothreitol	100	0.66
L-Ascorbic acid	100	0.12
Sodium sulphide	100	1.86
Methanol	200	1.44
Ethanol	200	1.62
Propanol	200	1.55

Table 2. *Effect on protoplast formation of treatment of mycelia with mercaptoethanol and ethanol prior to enzyme digestion*

Addition during:		$10^{-6} \times \text{No. of protoplasts ml}^{-1}$
Preincubation	Incubation	
—	Deionized water	0.21
—	2-Mercaptoethanol (100 mM)	1.60
—	Ethanol (200 mM)	1.53
Deionized water	—	0.94
2-Mercaptoethanol (100 mM)	—	0.91
Ethanol (200 mM)	—	1.35

deionized water without ethanol also considerably enhanced cell wall lysis. The optimal concentrations of mercaptoethanol and ethanol were 100 mM and 200 mM, respectively; higher concentrations decreased protoplast yields, due to protoplast lysis.

D-Sorbitol (0.5 to 0.7 M) was the best osmotic stabilizer for protoplast formation. A pH value of 7.0, which is optimal for Zymolyase activity, was most favourable for the formation of protoplasts.

Effect of mycelial age on protoplast formation

Newly isolated conidia and young germlings of *C. lamprauges* were resistant to the lytic enzymes and did not release protoplasts. However, germings with empty conidial shells and 20 h cultured mycelia were susceptible to the enzymes and were completely converted to protoplasts.

Fig. 3. Time course of protoplast formation in the standard reaction mixture. Micrographs after 0 min (a), 30 min (b), 60 min (c) and 120 min (d) incubation. Bars, 25 μ m.

Fig. 4. Time course of protoplast regeneration in soft ME agar medium. Micrographs after 0 h (a), 2 h (b), 4 h (c), 8 h (d), 20 h (e) and 40 h (f) incubation. Bars in (a–e), 25 μ m; bar in (f), 50 μ m.

Microscopic observation of protoplast formation

The time course of protoplast formation was observed microscopically under optimal conditions, i.e. with mycelia cultured for 20 h treated with a combination of Zymolyase 60000 and chitinase in the presence of 200 mM-ethanol. The mycelia were subdivided into small irregular fragments within 30 min of the start of incubation (Fig. 3). The fragments were transformed into distorted cells within a further 30 min, and after 2 h incubation almost all the cells were converted to spherical and osmotically labile protoplasts 7.5 to 20 μ m in diameter.

Regeneration of protoplasts

The protoplasts obtained readily regenerated hyphae when cultured in soft agar or in liquid media. The time course of regeneration in a soft ME agar is shown in Fig. 4. Morphological changes were detected as early as 2 h, when the surface of the protoplasts became irregularly shaped. Hyphal growth appeared clearly after 4 h and many extended hyphae and branchings were observed after 8 h. No budding and protrusion occurred in the soft ME agar. After 40 h incubation single protoplasts had developed into colonies which could be observed with the naked eye. The efficiency of regeneration was approximately 65% in this system, and was the same in soft MY agar and in liquid ME and MY media.

DISCUSSION

Protoplasts are useful for studies on cell wall biosynthesis and on the secretion process of extracellular and cell wall-bound enzymes, and for fusion in somatic cell genetics (Villanueva, 1966). We have studied exocellular and endocellular chitin-binding lectins of *C. lamprauges* (Ishikawa *et al.*, 1979, 1983). This fungus belongs to the Entomophthorales, which includes a number of insect pathogens (King, 1976). It contains chitin in the cell wall and produces exocellular chitinase and β -*N*-acetylglucosaminidase (Ishikawa *et al.*, 1981). Thus *C. lamprauges* is a good system for studying the mechanism of biosynthesis and biodegradation of chitin in fungi in relation to the physiological functions of chitin-binding lectins, chitinase, and β -*N*-acetylglucosaminidase. Since *C. lamprauges* formed protoplasts readily and regenerated hyphae at a high frequency and a high rate, we are attempting to use this organism to investigate the 'primer' which may be necessary for regeneration of chitin in the cell wall (Peberdy, 1979) and to screen new inhibitors of chitin biosynthesis in fungi.

Protoplasts were formed from older germlings and mycelia of *C. lamprauges* by treatment with Zymolyase (β -glucanase) and chitinase. Mannase was not required for protoplast formation, though mannose constitutes one-third of the sugars in the cell wall of this organism. Snail sulphatase is a potent enzyme for the lysis of yeast and fungal cell walls (Eddy, 1958, Ohnuki *et al.*, 1981); it released protoplasts from the conidia of *Neurospora crassa*, which are more resistant than vegetative forms (Bachmann & Bonner, 1959). In the present study, however, this enzyme was ineffective when used alone and was unnecessary in the presence of Zymolyase and chitinase.

Mercaptoethanol was required for the action of Zymolyase on living cells of *Candida lipolytica*; cysteine, sodium thioglycollate and sodium sulphide enhanced lysis to a lesser extent, whereas dithiothreitol, L-ascorbic acid and cysteamine were ineffective (Kaneko *et al.*, 1973). When young mycelia of *Geotrichum candidum* were treated with mercaptoethanol prior to the digestion by a lytic enzyme complex of *Streptomyces satsumaensis*, cell wall lysis was markedly accelerated (Dooijewaard-Kloosterziel *et al.*, 1973). The former but not the latter was also observed in the present study.

It is not clear whether the effect of lower alcohols was derived from their structural resemblance to mercaptoethanol. Treatment of *C. lamprauges* cells with those alcohols prior to enzyme digestion resulted in enhanced protoplast formation, but mercaptoethanol did not have this effect, suggesting that the mechanism of action of the alcohols was not identical to that of mercaptoethanol. It is possible that an autolytic reaction or selective inhibition of cell wall synthesis which is accelerated by the alcohols may participate in protoplast formation. Protoplast formation without added enzyme was reported in an osmotic mutant of *N. crassa* (Hamilton & Calvet, 1964).

High concentration of sugars may affect synthesis and autolysis of cell walls (Hamilton & Calvet, 1964; de Terra & Tatum, 1961). In the present study, however, because the protoplasts regenerated hyphae effectively in the presence of 0.5 to 0.7 M-D-sorbitol it presumably acted solely as an osmotic stabilizer.

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