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Total inhibition of wall synthesis by 2-deoxyglucose and polyoxin D in protoplasts of *Schizophyllum commune*

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SUMMARY

The synthesis of the alkali-soluble (1→3)-α-glucan in the wall of *Schizophyllum commune* (s-glucan) is completely and specifically inhibited by the addition of 2-deoxyglucose to regenerating protoplasts. In the presence of this substance the protoplasts were still able to generate hyphae indicating that the (1→3)-α-glucan does not play an essential role in hyphal morphogenesis. Specific inhibition of formation of chitin and alkali-insoluble wall glucan by polyoxin D prevented hyphal morphogenesis and led to osmotically stable cells containing walls essentially consisting of s-glucan only. Together, 2-deoxyglucose and polyoxin D prevented the protoplasts from forming any wall component.

Key-words: wall synthesis, 2-deoxyglucose, polyoxin D, protoplasts, *Schizophyllum commune.*

INTRODUCTION

It was shown previously that the alkali-soluble fraction of the hyphal wall of *Schizophyllum commune* (s-glucan) consists of (1→3)-α-glucan which is located predominantly at the outside of the wall as a partially crystalline material with a characteristic X-ray diffraction spectrum (Wessels *et al.* 1972). The alkali-insoluble fraction of the wall is mainly composed of a (1→3)-β/(1→6)-β-glucan (R-glucan) covalently linked to chitin (Sietsma & Wessels 1979). The linkage between these polymers was suggested by the findings that (a) degradation of chitin by specific enzymatic or chemical treatments solubilized the glucan and (b) synthesis of the alkali-insoluble glucan was shown to result from linking a water-soluble/alkali-soluble glucan from a precursor-pool to newly synthesized alkali-insoluble chitin (Sonnenberg *et al.* 1982; Wessels *et al.* 1983).

On the basis of these results a model of hyphal morphogenesis was proposed in which the initially synthesized wall possessed visco-elastic properties and expands under turgor pressure. The tubular hyphal form is fixed in subapical regions by processes involving linking of glucan to chitin giving the wall its final rigid structure (Wessels 1986, 1988). The alkali-soluble (1→3)-α-glucan was not implicated as playing a role in the generation of the hyphal form. The present study supports this model and shows that inhibition of (1→3)-α-glucan synthesis by 2-deoxyglucose during protoplast regeneration has no effect on the regeneration of hyphae. Hyphal morphogenesis

was prevented by polyoxin D which interfered with the synthesis of the glucan–chitin complex. Polyoxin D and 2-deoxyglucose together prevented synthesis of all wall components.

MATERIALS AND METHODS

Growth of mycelium and preparation of protoplasts

Dikaryotic fruiting mycelium of *Schizophyllum commune* was obtained by mating strains 1–40 (CBS 344.81) and 1–50 (CBS 342.81). Protoplasts were derived from germinated basidiospores, isolated and purified as described before (Sonnenberg *et al.* 1982).

Regeneration of protoplasts and isolation of polysaccharide fractions

Protoplasts (10^6/ml) were suspended in regeneration medium consisting of minimal medium (Wessels 1965) containing 10 mg/ml glucose supplemented with 0·5 M $MgSO_4$ and incubated under aerobic conditions (in shallow layer) at 25°C. 2-Deoxyglucose (Sigma, St Louis, MI, USA) at various concentrations and polyoxin D (a gift from Kaken Chemical Company, Tokyo, Japan) at 50 µl/ml were added at the start of regeneration. Samples were taken at intervals and regeneration was stopped by addition of 1 vol. 10% (w/v) trichloracetic acid (TCA). The precipitate was collected by centrifugation, washed with water and extracted with 1 M KOH at 60°C for 20 min under N_2. (1→3)-α-glucan was precipitated by acidification of the alkaline extract with acetic acid, recovered by centrifugation, and dissolved in 0·5 M KOH (Wessels *et al.* 1972). The alkali-insoluble β-glucan was solubilized by treating the alkali-insoluble residue with 0·55 M HCl at 100°C for 1 h under N_2 and subsequently with 1 M KOH at 60°C for 20 min (Sietsma & Wessels 1977). The chitin in the insoluble residue was hydrolysed with 6 M HCl at 120°C for 2 h under N_2 in closed tubes, the hydrolysate was dried in a desiccator over KOH and P_2O_5 and dissolved in water (Sonnenberg *et al.* 1982).

Chemical essays

Glucan in the different fractions was estimated using the anthrone reagent (Fairbairn 1953). The values obtained for the HCl and subsequent KOH extraction were added together for alkali-insoluble glucan. Chitin was determined by analysing glucosamine in the hydrolysed fractions by the procedure of Johnson (1971).

RESULTS

The effect of 2-deoxyglucose on (1→3)-α-glucan synthesis

Previous studies (de Vries & Wessels 1975, van der Valk & Wessels 1976, Sonnenberg *et al.*, 1982) have shown that protoplasts of *Schizophyllum commune*, when suspended in minimal medium with 10 mg/ml glucose and with 0·5 M $MgSO_4$ as osmotic stabilizer, are capable of regenerating a normal cell wall and eventually producing normal hyphae. When, during regeneration, the accumulation of individual polymers was followed, it was found that the synthesis of (1→3)-α-glucan and chitin was at a constant rate from the start of regeneration, but synthesis of the alkali-insoluble glucan showed a lag period of 3 h (de Vries & Wessels 1975, Sonnenberg *et al.* 1982). Similar results were obtained in the present study (Figs 1b and 2a). When 2-deoxyglucose was added to the regeneration medium in increasing amounts, the synthesis of (1→3)-α-glucan was progressively inhibited (Fig. 1a).

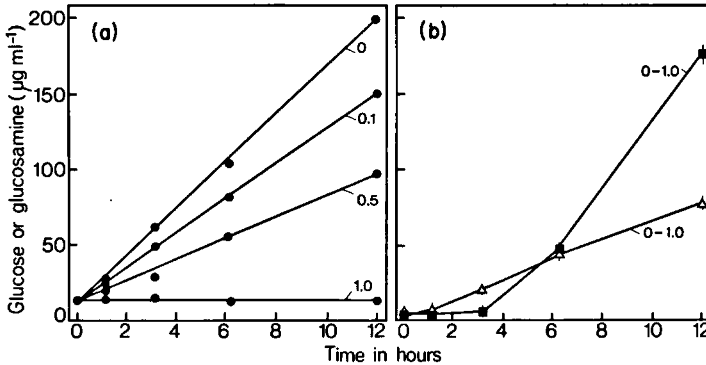

Fig. 1. Accumulation of glucans (measured as anthrone-positive material) and chitin (measured as glucosamine) during regeneration of protoplasts of *Schizophyllum commune* in the absence or presence of 2-deoxyglucose. Next to the curves the amount of 2-deoxyglucose (mg/ml) is indicated. In (b) the values at various 2-deoxyglucose concentrations are averaged and fall within the range indicated by the vertical lines. (●) S-glucan, (■) R-glucan, (△) chitin.

Neither the formation of the alkali-insoluble glucan nor the synthesis of chitin seemed to be affected by the addition of 2-deoxyglucose (Fig. 1b).

The effects of 2-deoxyglucose and polyoxin D on the synthesis of the various cell wall polymers and on morphological regeneration

When 2-deoxyglucose was added to the regeneration medium in a concentration of 1 mg/ml, the synthesis of the alkali-soluble glucan [(1→3)-α-glucan] was completely inhibited, while the synthesis of both the other cell wall polymers was unaffected (Fig. 2a and b). This total inhibition of (1→3)-α-glucan synthesis did not seem to have any effect on the generation of the hyphal form because after 12 h regeneration in the presence of 1 mg/ml 2-deoxyglucose normal hyphae appeared (Fig. 3b). The protoplasts also became osmotically stable; transfer to distilled water after 3 h of regeneration did not cause bursting.

Polyoxin-D, a specific inhibitor of chitin synthesis, added to the medium at a concentration of 50 μg/ml during the regeneration of *Schizophyllum commune* protoplasts, inhibited the accumulation of both chitin and alkali-insoluble glucan (R-glucan) but not that of the alkali-soluble (1→3)-α-glucan (S-glucan) (Fig. 2c). Microscopically it was evident that in the presence of polyoxin-D the protoplasts remained spherical and no hyphae were formed (Fig. 3d). However, the protoplasts continued to increase in size and retained the tendency to clump together (Fig. 3f) to form large aggregates. This has been reported to be the first visible sign of wall regeneration (De Vries & Wessels 1975).

When both inhibitors, polyoxin D and 2-deoxyglucose, were added to the regeneration medium, the synthesis of all three cell wall fractions was completely inhibited (Fig. 2d) and the protoplasts remained osmotically sensitive. After 12 h in regeneration medium, dilution of the medium with 1 volume of distilled water caused bursting. Microscopically no hyphae were detected and the protoplasts did not aggregate (Fig. 3c). They did remain viable, however and continued to increase in size (Fig. 3e); their diameter increased about five times during a 12-h incubation period.

DISCUSSION

The present study shows that 2-deoxyglucose at a concentration of 1 mg/ml in the presence of 10 mg/ml glucose causes complete inhibition of (1→3)-α-glucan synthesis in

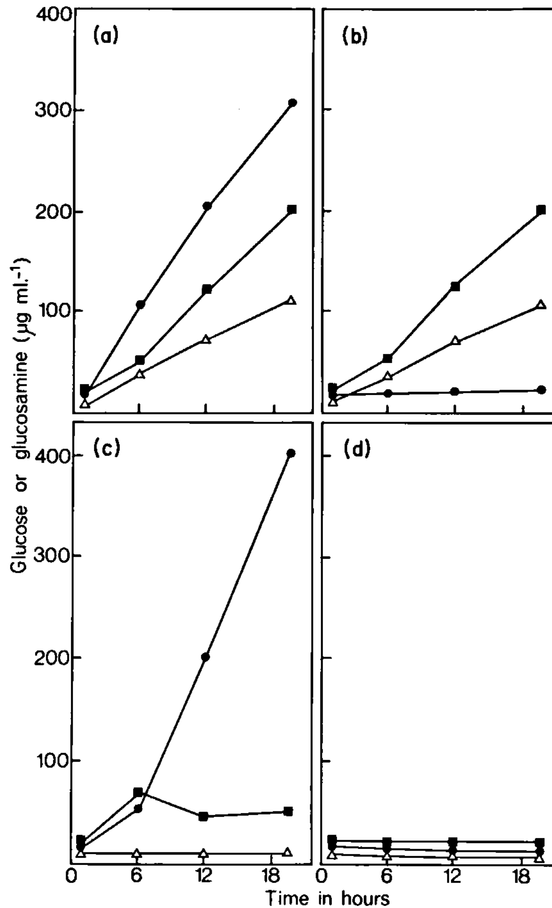

Fig. 2. Accumulation of glucans (measured as anthrone-positive material) and chitin (measured as glucosamine) during regeneration of protoplasts of *Schizophyllum commune* in the absence or presence of 2-deoxyglucose and/or polyoxin D. (a) Normal regeneration; (b) regeneration in the presence of 1 mg/ml 2-deoxyglucose; (c) regeneration in the presence of 50 μg/ml polyoxin D; (d) regeneration in the presence of both 2-deoxyglucose (1 mg/ml) and polyoxin D (50 μg/ml). (●) S-glucan, (■) R-glucan, (△) chitin.

regenerating protoplasts of *Schizophyllum commune*, without affecting the synthesis of the other wall components (1→3)-β-/(1→6)-β-glucan and chitin. A specific inhibition of (1→3)-α-glucan synthesis by 2-deoxyglucose has also been noted by Zonneveld (1973) in *Aspergillus nidulans*. The significance of the present study is that complete inhibition of (1→3)-α-glucan synthesis does not interfere with the normal outgrowth of hyphae from regenerated protoplasts, indicating that this glucan, which makes up about 30% of the mature wall of *Schizophyllum commune* (Sietsma & Wessels 1977), plays no essential role in hyphal morphogenesis. This was also suggested by the work of Zonneveld (1974) and Polacheck & Rosenberger (1977) who studied mutants of *Aspergillus nidulans* containing little or no (1→3)-α-glucan in their walls. Although no information was given on hyphal morphology, the mutants grew on normal medium and apparently had rigid walls. On the other hand, for the formation of protoplasts from *Schizophyllum commune* hyphae, (1→3)-α-glucan has to be degraded (de Vries & Wessels 1973). Also hyphae of *Schizophyllum*

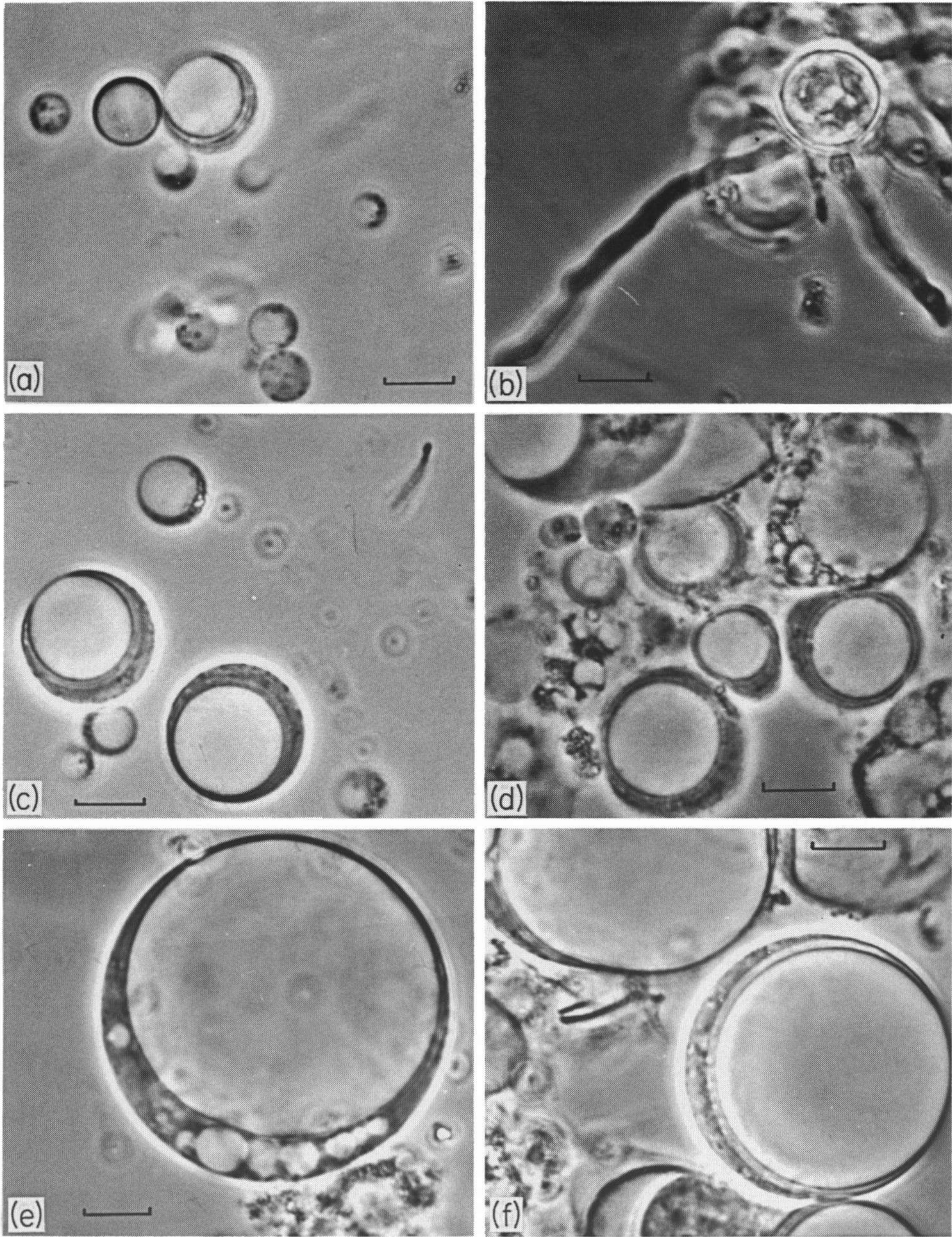

Fig. 3. Regeneration of *Schizophyllum commune* protoplasts in the absence or presence of 2-deoxyglucose and/or polyoxin D, viewed by fase-contrast microscopy; bar represents 10 µm. (a) Protoplasts at the start of the regeneration process. (b) Regeneration of proptoplasts after 12 h in the presence of 2-deoxyglucose (1 mg/ml). (c) Protoplasts after 6 h regeneration in the presence of 2-deoxyglucose (1 mg/ml) and polyoxin D (50 µg/ml). (d) Protoplasts after 6 h regeneration in the presence of polyoxin D (50 µg/ml). (e) Giant protoplast observed after 12 h regeneration in the presence of 2-deoxyglucose (1 mg/ml) and polyoxin D (50 µg/ml). (f) Cluster of giant protoplasts after 12 h regeneration in the presence of polyoxin D (50 µg/ml).

commune, from which most of the components, apart from (1→3)-α-glucan, have been removed by autolytic processes, retain their normal form (Wessels 1965; Wessels & Sietsma 1979). It thus appears that this glucan, although not essential for hyphal morphogenesis, may be responsible for the maintenance of hyphal morphology in mature hyphae.

It has been shown that 2-deoxyglucose also has an effect on cell wall mannan and glucan formation in yeast and also influences protein secretion (Johnson 1968; Kuo & Lampen 1972). In general it seems to interfere with protein glycosylation and the linkage of mannose residues to the dolichol intermediate seems especially to be affected (Datema & Schwartz 1979). However, the specific effects of 2-deoxyglucose are difficult to study since it is an analogue of glucose and might affect the total cellular machinery by interfering with normal sugar metabolism(Biely *et al.* 1972). Therefore, it is remarkable that in the regeneration of protoplasts of *Schizophyllum commune*, 2-deoxyglucose affected only the synthesis of one of the three main wall polymers which are all ultimately derived from glucose. Unfortunately nothing is known about the mechanism of (1→3)-α-glucan synthesis in fungi. The effect of 2-deoxyglucose on the synthesis could suggest a lipid or glycoprotein intermediate although we found that *in vivo,* tunicamycin had no effect on the synthesis of this glucan (Sietsma, unpublished observations).

As shown earlier (de Vries & Wessels 1975) polyoxin D inhibited the accumulation of both chitin and alkali-insoluble glucan. Sonnenberg *et al.* (1982) have shown that the inhibition of formation of alkali-insoluble glucan is not due to the inhibition of synthesis of the β-glucan chains but to the absence of chitin chains to which they can be linked. In the presence of polyoxin D there was also no inhibition of the accumulation of the alkali-soluble (1→3)-α-glucan (de Vries & Wessels 1975, van der Valk & Wessels 1976). Recently, Elorza *et al.* (1987) have also shown that the specific inhibition of chitin synthesis by nikkomycin not only inhibits the synthesis of chitin but, in addition, the conversion of an alkali-soluble glucan into an alkali-insoluble form on protoplasts of *Candida albicans*.

The effects of polyoxin D and 2-deoxyglucose on regenerating protoplasts, as reported here, indicate that the chitin–β-glucan complex but not the (1→3)-α-glucan may play an essential role in the generation of hyphal morphology. This would agree with the role attributed to the chitin–β-glucan complex in the steady-state model for apical wall growth (Wessels 1986, 1988).

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