Effect of Lactose on Soluble-glucan Production and on the Ultrastructure of *Sclerotium rolfii* Sacc. Grown in Submerged Culture

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SUMMARY

A significant increase in mycelial dry weight and a decrease in production of extracellular glucan were observed when lactose was added to a submerged culture of *Sclerotium rolfii* grown in a liquid glucose-synthetic medium. When added as a sole carbon source, lactose at 2.5 % (w/v) induced the formation of dark, spherical, compact bodies. The ultrastructure of these bodies is compared with that of sclerotia formed on solid media.

INTRODUCTION

Usually sclerotium formation and sporulation in fungi takes place on the surface of solid media rather than in submerged cultures. Submerged conidiation or sclerotium formation would greatly facilitate the investigation of biochemical events occurring during differentiation. The conidiation of *Aspergillus niger* in submerged culture has been reported by Galbraith & Smith (1969). Chet & Rusch (1969) induced spherule formation in the slime mould *Physarum polycephalum* by adding 0.5 M-mannitol to a synthetic liquid medium. Addition of lactose to the growth medium resulted in synchronous formation of sclerotia in *Sclerotium rolfii* (Okon, Chet & Henis, 1972), sclerotia being formed at the edge of the growing colony. The purpose of this study was to examine the effect of lactose on *S. rolfii* in submerged culture.

METHODS

Growing conditions and chemical analysis. *Sclerotium rolfii* was grown in 250 ml Erlenmeyer flasks containing 50 ml synthetic medium (SM) (Okon, Chet & Henis, 1973). These were inoculated with mycelial discs, 1 cm diam, obtained from a colony of *S. rolfii* grown on solidified SM, and the flasks were shaken (120 strokes/min) for 7 days at 30 °C. The culture was centrifuged at 800 g for 10 min and the propagules washed three times by centrifugation with sterile tap water. The inoculum prepared from this material and used for all other experiments was a 2 ml fungal suspension containing 20 mg dry wt. After 7 days, cultures were centrifuged at 800 g for 10 min and the mycelium was washed three times in tap water, dried at 80 °C for 24 h, and weighed. Wall material was obtained by the method described by Chet, Henis & Mitchell (1967). Water-soluble extracellular polysaccharides were precipitated by centrifugation after adding 2 vol. of cold ethanol to the mycelium-free medium. The precipitate was washed three times with cold ethanol, lyophilized, and weighed. Twenty mg of either extracellular polysaccharide or mycelial walls were hydrolysed in 2 ml of 4 N-HCl at 100 °C for 5 h. The hydrolysate was neutralized,

Fig. 1. Effect of lactose added to a synthetic liquid medium on mycelial dry weight and soluble-polysaccharide production by *Sclerotium rolf sii*. ▲, Polysaccharide; ●, dry weight.

and analysed for reducing sugars by descending paper chromatography with benzene: butanol:pyridine:water (1:5:3:3, by vol.) as a running solvent, and with AgNO_3 (1 % in acetone solution) and NaOH (2 % in ethanol solution) as developing reagents. Glucose was determined by a glucostat reagent (Worthington Co., New Jersey, U.S.A.) and glucosamine was measured according to Elson & Morgan (1933). All experiments were carried out three times, each in five replicates.

Transmission electron microscopy (TEM). Cultures of *Sclerotium rolf sii* were fixed for 2 h in cold 5 % glutaraldehyde in 0.1 M-phosphate buffer (pH 7.0), rinsed with buffer, and post-fixed for 20 h with 2 % osmium tetroxide in the same buffer. In some experiments the samples were fixed for 1 h at room temperature with a mixture (1:1, v/v) of freshly prepared 7 % glutaraldehyde and 4 % osmium tetroxide in 0.1 M-phosphate buffer (pH 7.0). The fixed material was rinsed, dehydrated with ethanol and embedded in Epon 812. Thin sections were prepared with an LKB Ultratome III, stained with uranyl acetate and lead citrate and observed in a Y.E.M.T. 7 electron microscope. Thick sections were cut in the same way, stained with toluidine blue, and observed by light and phase microscopy.

Scanning electron microscopy (SEM). Samples from submerged cultures were blotted and attached to the microscope specimen stubs. For comparison, sclerotia of different ages from cultures grown on Petri dishes were taken and treated in the same way. The samples were frozen in liquid nitrogen for a few minutes and lyophilized for 3 h. Some samples were fixed (before freezing and drying) in the same way as material prepared for TEM. For observations of internal structures, samples from shaken flasks were opened with needles, while sclerotia were cut with a razor blade before freezing and drying. The dried samples were coated with a thin layer of gold and observed in a Cambridge Stereoscan electron microscope (SEM) S4 at 10 or 20 kV.

RESULTS

Increasing lactose concentration in the growth medium increased mycelial dry weight and lowered production of extracellular glucan (Fig. 1). The addition of lactose to the growth medium also resulted in the colour of the mycelium changing from white through yellow to dark brown.

Chromatographic and chemical analysis of the hydrolysed extracellular polysaccharide and mycelial wall of all preparations revealed the presence of glucose and glucosamine but not galactose. No significant differences in the ratio of glucose to glucosamine were observed in the tested preparations. Sclerotium formation was not seen even after growing cultures for 2 months in shaken flasks containing media used to induce conidiation in *Aspergillus niger* (Galbraith & Smith, 1969) or spherule formation in *Physarum polycephalum* (Chet & Rusch, 1969).

When the fungus was grown for 2 weeks on SM containing lactose (2.5 %, w/v) as sole carbon source, growth was poor (40 mg total dry weight as compared with 275 mg in the glucose medium). On this medium, however, *Sclerotium rolfii* produced dark, spherical, compact structures, 1 to 3 mm diam (Fig. 2) in contrast to big white propagules formed on SM (Fig. 3). The SEM revealed that their structure was different from that of sclerotia obtained from a culture grown on solidified SM (compare Fig. 2 and 3 to Fig. 4 and 5). Lactose-grown cultures had a central mass of cells surrounded by loosely interwoven hyphae (Fig. 2).

Immature (white) and mature (brown-black) sclerotia kept their rounded structure even when desiccated at room temperature (Fig. 4 and 5). Observation of thick sections by light and phase-contrast microscopy revealed mycelium built of loosely arranged short hyphae with many branches. Large rounded cells, 6 to 20 µm in diam, were found scattered in the compact bodies (Fig. 6). There were far fewer in controls without lactose. TEM photographs showed that most of the large cells were empty or had very large vacuoles and only a thin layer of cytoplasm (Fig. 7).

DISCUSSION

Cultures of *Sclerotium rolfii* grown on a lactose-supplemented solid synthetic medium showed increased hyphal branching (Henis, Okon & Chet, 1973) and synchronous sclerotium formation (Okon *et al.* 1972). In submerged cultures, lactose increased mycelial dry weight and decreased the production of an extracellular soluble glucan.

Tatum, Barratt & Cutter (1949) found that sorbose restricted the size of fungal colonies in *Neurospora* by restricting apical growth of hyphae and increasing the degree of branching. In the fungus *Podospora anserina*, sorbose, galactose and 2-D-deoxyglucose inhibited elongation and enhanced branching of hyphae (Lysek & Esser, 1971), probably as a result of competitive inhibition of polysaccharide synthesis by these sugars (Johnson, 1968; Lysek & Esser, 1971). Addition of glucose inhibited the effect of both lactose (Okon *et al.* 1972) and galactose (Lysek & Esser, 1971). This leads to the assumption that lactose acts similarly as a competitive inhibitor of β -D-glucan production.

Sclerotium rolfii, grown in submerged cultures containing lactose, produced compact spherical structures built of interwoven mycelium. When grown on solid SM, the fungus formed brown, hard-walled sclerotia consisting of organized layers of cells (Chet, Henis & Kislev, 1969). The morphology of the compact bodies reminds one of sclerotia, but microscopical observation showed no typical organization.

The many unusually large cells observed on these bodies were similar to those found in the fungus *Dendryphiella salina* (Holligan & Jennings, 1972). So far, the relationship between sclerotia and the compact structure obtained under submerged conditions in lactose-containing medium, as well as the reason(s) for the inability of *Sclerotium rolfii* to form typical sclerotia in submerged conditions remain obscure. Oxygen supply and relative humidity are probably involved and deserve further investigation.

Fig. 2. Scanning electron microscope (SEM) photograph of a compact body of *Sclerotium rolfsii* grown in lactose supplemented liquid medium.

Fig. 3. SEM photograph of part of mycelial propagule grown in liquid SM.

Fig. 4. SEM photograph of young sclerotium grown on solid SM.

Fig. 5. SEM photograph of mature sclerotium grown on solid SM.

Fig. 6. SEM photograph of broken big cell in the compact body.

Fig. 7. Transmission electron microscope photograph of section of big cell from the compact body. N, nucleus; V, vacuole; C, cytoplasm; W, cell wall.

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