Distribution of (1→3)- β - and (1→4)- β -Glucan Synthases Along the Hyphae of *Saprolegnia monoica*

By VINCENT GIRARD AND MICHEL FÈVRE*

Laboratoire de Mycologie associé au CNRS no. 44, Université Claude Bernard, Bâtiment 405,
43 Bvd du 11 novembre 1918, 69622 Villeurbanne Cédex, France

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Protoplasts from hyphae of *Saprolegnia monoica*, collected from lytic medium after different times of incubation, represented a sequential fractionation of mycelium from apical to distal zones. This method gives the opportunity to study the intrahyphal localization of cell wall glucan synthases in relation to apical growth. (1→4)- β -Glucan synthases were mainly found in early protoplasts (i.e. from apical zones) while the highest (1→3)- β -glucan synthase activities were recovered in late protoplasts (i.e. from the subapical zones). Early protoplasts also exhibited the highest rate of cell wall synthesis during regeneration. The distribution of the enzymes along the fungal hyphae might provide a clue to the mechanisms of cell wall polysaccharide deposition during apical growth.

INTRODUCTION

Cell walls of Oomycetes, such as *Saprolegnia*, possess cellulose and (1→3)- β /(1→6)- β -glucans which represent respectively 21 and 79% of the cell wall carbohydrates (Sietsma, 1969). The cell wall construction is similar at the apex and in subapical zones: the outer layer, constituted by amorphous glucans containing (1→3)- β /(1→6)- β linkages, overlays the inner layer of microfibrillar cellulose (Hunsley & Burnett, 1970; Beckett *et al.*, 1974). However, the cell wall of the apex is thinner than in the older regions of the hyphae and, in the apical region, the outer glucan layer is very thin (Hunsley, 1973).

In the present study, protoplasts were used as a system to study intrahyphal localization of cell wall glucan synthases. Suspensions of fungal protoplasts are heterogeneous in relation to their original hyphal location. Protoplast production during the lysis of mycelium has been considered as a sequential release of cytoplasm from apical to subapical zones (Isaac *et al.*, 1978). In *Saprolegnia monoica*, the 'early' protoplasts were mostly anucleate while 'late' protoplasts contained one or more nuclei (Gaugy & Fèvre, 1982). In *Aspergillus nidulans*, an ultrastructural analysis showed that the protoplasts produced at the beginning of lysis exhibited the cellular organization of the apical cytoplasm of the hyphae. Protoplasts produced after a prolonged incubation had characteristics of the older parts of the hyphae (Isaac *et al.*, 1979).

The harvesting of protoplasts at different times of digestion can be considered as a dissection of the hyphae in successive zones from the apical to the distal regions. In this work, we have used this method of studying the cell wall polysaccharide synthase activities of different samples of protoplasts, thus reflecting the localization of these enzymes along the hyphae of *Saprolegnia*.

METHODS

Culture methods. *Saprolegnia monoica* Pringsheim (no. 539 67 Dick) obtained from CBS, Baarn, The Netherlands, was maintained on a wheat flour medium. Mycelia were grown in liquid medium (Machlis, 1953) at 23 °C for 48 h and harvested by filtration.

Protoplast production. Mycelia (48 h old) were converted to protoplasts during incubation in a lytic medium pH 5.8, containing 5 mg Driselase ml⁻¹ (Fluka, Buchs, Switzerland), 1 mg cellulase ml⁻¹ and 0.5 M-sorbitol as

stabilizer (Gaugy & Fèvre, 1982). Phenylmethylsulphonyl fluoride (120 mM) was included in the mixture as it permits recovery of higher synthase activities. At different times of incubation, protoplasts were collected by filtration through bolting cloth (70 μm) and non-digested mycelium was again incubated in a fresh lytic medium. Protoplasts of the different samples were submitted to equivalent treatment as they were kept in the lytic medium until the end of the last incubation. Protoplasts were separated from digestive enzymes by centrifugation at 700 g for 10 min, washed twice with the osmotic medium, and then resuspended at a final concentration of 10^6 to 10^7 protoplasts ml^{-1} .

Preparation of particulate enzymes. Protoplasts were mixed in buffer (0.01 M-Tris/HCl, 0.5 M-sorbitol, 0.001 M-EDTA, pH 7.2) containing 1% (w/v) dithiothreitol, using a virtis homogenizer from three periods of 30 s. Alternatively, protoplasts were broken by slow passage through a syringe and then through a glass homogenizer with a Teflon pestle. Membranes were collected by centrifugation at 48 000 g for 30 min.

Fixation of concanavalin A on protoplasts: isolation of the plasma membrane fraction from concanavalin A-labelled protoplasts. Protoplasts were suspended in Tris/HCl buffer pH 7.4, containing 0.5 M-KCl, 0.01 M- CaCl_2 and 250 μg concanavalin A ml^{-1} . The mixture was maintained at 4 °C for 30 min. Non-fixed concanavalin A was eliminated by dilution of the protoplast suspension with buffer, then centrifugation. The pellet was washed twice with buffer, then resuspended in 10 mM-Tris/HCl buffer pH 7.4, containing 0.1 M-KCl and 0.01 M- CaCl_2 . The suspension was homogenized as described above. The plasma membrane fraction was collected by centrifugation of the lysate at 500 g for 10 min. The supernatant was centrifuged at 48 000 g for 30 min, to pellet the internal membranes.

Enzyme assays. When enzymes were assayed for (1 $\rightarrow$ 4)- β -polysaccharide synthesis, the complete mixture (total volume 370 μl) contained 0.3 nmol UDP[^{14}C]glucose (7.4 mBq μmol^{-1}), 0.1 μmol dithiothreitol, 2 μmol cellobiose, 5.5 μmol MgCl_2 , 25 mM-PIPES/Tris buffer pH 6.0 and 100 μl freshly isolated enzymes. When enzymes were assayed for (1 $\rightarrow$ 3)- β -glucan synthesis, MgCl_2 was replaced by 400 nmol UDP[^{14}C]glucose. Mixtures were incubated at 27 °C for 30 min. Reactions were terminated by heating the tubes for 2 min in a boiling bath or by addition of 2 ml ethanol and precipitation overnight at -20 °C. After addition of powdered cellulose (10 to 20 mg), mixtures were filtered through glass fibre filters (Whatman GF/C 2.4 cm). Residues on the filter were washed with 40 ml water, 20 ml chloroform/methanol (2:1, v/v) and 40 ml ethanol. Radioactivity of filters was determined with an Intertechnique SL 33 scintillation counter, using a PPO/POPOP/toluene scintillation mixture.

Protoplast regeneration. Protoplasts were suspended in the culture medium of Machlis (1953) containing 0.5 M-sorbitol, 0.1% glucose and 1 μCi (37 kBq) [^{14}C]glucose ml^{-1} (0.9 GBq mmol^{-1}). Under these conditions, a complete cell wall was reconstituted after 6 h regeneration as revealed by electron microscopy (Girard, 1983). The suspension (1 to 5×10^{-6} protoplasts ml^{-1}) was incubated at 24 °C in a shaking incubator. Samples were taken at intervals and the regeneration process stopped by adding 2 vol. ethanol. Suspensions were precipitated overnight at -20 °C.

Isolation of polysaccharide fractions. After ethanol precipitation, protoplasts were collected by centrifugation and washed with water, chloroform/methanol (2:1, v/v) and ethanol; the remaining material represented the total cell wall polysaccharides of regenerating protoplasts. The cellulose fraction was isolated according to Updegraff (1969). After centrifugation, ethanol-precipitated protoplasts were resuspended in acetic/nitric acid reagent (80% acetic acid/nitric acid; 10:1, v/v) and placed in a boiling water bath for 30 min. Non-hydrolysable material, representing the cellulose fraction of regenerated cell walls, was collected by filtration. Radioactivity was determined as described above.

RESULTS

Glucan synthase activities in protoplasts

Protoplasts collected after different times of production (20, 40, 60, 90 and 120 min) were washed then homogenized. Glucan synthases were assayed on membranes collected after centrifugation at 48 000 g for 30 min (Fig. 1). (1 $\rightarrow$ 4)- β -Glucan synthase activity was highest in protoplasts released after 20 min digestion. The activity decreased in the following samples and represented 50% of the maximal activity in 'late' protoplasts collected after 120 min of lysis.

The distribution of (1 $\rightarrow$ 3)- β -glucan synthase activity in the different protoplast samples was the opposite. Synthesis activity, low in the initial samples, increased in protoplasts obtained after long incubation - maximal activity (four times the activity detected in the first sample) was observed in protoplasts harvested after 90 min of lysis.

However, (1 $\rightarrow$ 3)- β -glucan synthase activity, even at its lower level, was much greater than (1 $\rightarrow$ 4)- β -glucan synthase activity. In the first sample of protoplasts, 20 nmol glucose were incorporated in conditions of (1 $\rightarrow$ 3)- β -glucan synthesis, as compared with 0.025 nmol in conditions of (1 $\rightarrow$ 4)- β -glucan production. These low values observed for (1 $\rightarrow$ 4)- β -glucan

Fig. 1. Glucan synthase activities of cell free extracts of protoplasts released at different times of incubation. ■, (1→4)-β-Glucan synthase; ▲, (1→3)-β-glucan synthase. 0.1 kBq represents a glucose incorporation of 13 nmol in conditions of (1→3)-β-glucan synthesis and 0.01 nmol in conditions of (1→4)-β-glucan synthesis.

synthesis are comparable to those obtained with green plants (Raymond *et al.*, 1978; Henry & Stone, 1982; Langebartels *et al.*, 1981).

Glucan synthase activities of plasma membranes and intracellular membranes of protoplasts

Protoplasts collected after different times of mycelial digestion (20, 40, 60, 90 and 120 min) were labelled with concanavalin A before lysis. After breakage, protoplast extracts were fractionated by centrifugation into two fractions, a plasma membrane fraction and an intracellular membrane fraction, according to the procedure previously described (Girard & Fèvre, 1984).

(1→4)-β-Glucan synthase activity of the plasma membrane showed maximal activity in the first sample (i.e. 20 min lysis) and decreased in the other samples. The enzyme activity associated with intracellular membranes was highest in the second sample (i.e. 40 min incubation) and four times higher than the activity detected in the other samples (Fig. 2).

(1→3)-β-Glucan synthases exhibited a low activity in plasma membranes and intracellular membranes from the first sample. The activity of both fractions increased in protoplasts produced after longer incubation. In the later sample (120 min incubation), plasma membrane and intracellular membrane activities were respectively six to eight times higher than those found in the membranes of the first sample. The presence of trypsin, which increases *in vitro* (1→3)-β-glucan synthesis by activating enzymes (Fèvre, 1979), produced a strong stimulation of the synthase activities associated with internal membranes. This effect was particularly marked in membranes originating from 'late' protoplasts (fourfold stimulation). Enzymes associated with plasma membranes were only slightly stimulated by trypsin (Fig. 2).

Cell wall synthesis during protoplast regeneration

As the samples of protoplasts produced after different times of digestion exhibited differences in their glucan synthase activities, it was of interest to know if the pattern of cell wall production during regeneration followed the same trend.

Protoplasts collected after 30, 60, 90 and 120 min lysis were regenerated for 6 h in the presence of [¹⁴C]glucose (1 μCi ml⁻¹). Glucose incorporation was followed in the total cell wall polysaccharides and in a 'cellulose' fraction isolated according to the method of Updegraff (1969).

In the first sample, radioactivity incorporation into total cell wall polysaccharides increased regularly from the start of regeneration. The cellulose fraction, initially produced at a high rate, reached a plateau after 3 h regeneration (Fig. 3a). In the other samples of protoplasts, the incorporation of glucose in the different polysaccharides was lower: the later the protoplasts were produced, the lower the rate of incorporation. For example, total cell wall synthesis after

Fig. 2. Glucan synthase activities of the plasma membrane fraction (PMF) and of the internal membrane fraction (IMF) of protoplasts collected at different incubation times. ■, (1→4)-β-glucan synthase; ▲, △, (1→3)-β-glucan synthase assayed in the absence (▲) or presence (△) of 500 μg trypsin ml⁻¹. 0.1 kBq represents a glucose incorporation of 13 nmol in conditions of (1→3)-β-glucan synthesis and 0.01 nmol in conditions of (1→4)-β-glucan synthesis.

Fig. 3. [¹⁴C]Glucose incorporation into cell wall polysaccharides during regeneration of protoplasts released at different times of incubation: (a) 30 min; (b) 60 min; (c) 90 min; (d) 120 min. ●, Total cell wall polysaccharides; ○, cellulose fraction extracted according to Updegraff (1969).

6 h regeneration was respectively 3, 1.5, 0.8 and 0.5 kBq for the protoplasts collected after 30, 60, 90 and 120 min of lysis. However, in each case, cell wall synthesis was linear at the beginning of regeneration and then reached a plateau or even decreased. This trend was particularly noticeable for the synthesis of the cellulose fraction (Fig. 3b, c, d).

Fig. 4. Glucan synthase activity and glucan synthesis of protoplasts released at different times of incubation. Glucan synthase activity corresponds to the incorporation of glucose from UDPglucose into polysaccharides by the plasma membrane fraction of protoplast extracts (i.e. glucose incorporated *in vitro*). Glucan synthesis corresponds to the incorporation of radioactive glucose into cell wall glucans of intact protoplasts after 6 h regeneration (i.e. glucose incorporated *in vivo*). Results are expressed as the percentage of the total of the activity exhibited by the different samples of protoplasts. ■, (1→4)- β -Glucan synthase activity; ▲, (1→3)- β -glucan synthase activity; ●, total cell wall polysaccharide synthesis; ○, cellulose fraction synthesis.

DISCUSSION

The digestion of a fungal colony by lytic enzymes enables us to obtain a sequential fractionation of mycelium. Early protoplasts originate from apical cytoplasm and the protoplasts released later represent subapical and distal cytoplasm (Isaac *et al.*, 1978; Isaac & Gokhale, 1983; Gaugy & Fèvre, 1982). Thus, our results demonstrate that (1→3)- β - and (1→4)- β -glucan synthases have a different subcellular localization along the hyphae. (1→4)- β -Glucan synthases, found mainly in early protoplasts, must be located at the apex while (1→3)- β -glucan synthases recovered in late protoplasts must be associated with subapical cytoplasm. Plasma-membrane-bound (1→3)- β -glucan synthases are mostly active enzymes but the enzymes associated with internal membranes are in an inactive form which can be activated by moderate proteolysis with trypsin (Fèvre, 1979).

Delmer (1977) has suggested that in green plants (1→3)- β -glucans produced as a wound response would be synthesized by (1→4)- β -glucan synthases going wrong. Our results indicate that in fungi different enzyme systems must be involved in (1→3)- β - or (1→4)- β -glucan synthesis as the enzymes have a different location in the membranes and in the hyphae.

Regeneration of protoplasts showed that *in vivo* cell wall polysaccharide synthesis did not correlate with the distribution of glucan synthase activities assayed *in vitro*. The successive samples of protoplasts were capable, at different levels, of transferring *in vitro* glucose from UDPglucose to produce (1→3)- β - or (1→4)- β -polysaccharides. However, according to their time of release (i.e. their position in the hyphae), intact protoplasts did not show the same ability to incorporate glucose into cell wall polysaccharides. Early protoplasts, which had the highest (1→4)- β -glucan synthase activity, also showed the highest rate of glucose incorporation during cell wall regeneration. On the other hand, late protoplasts rich in (1→3)- β -glucan synthases had only a weak capacity to regenerate cell wall polysaccharides. Polysaccharide synthesis during regeneration of the different samples of protoplasts followed the distribution of plasma membrane (1→4)- β -glucan synthase activity among these different samples (Fig. 4). The

capacity of protoplasts to regenerate cell walls, which decreased from early protoplasts (apical cytoplasm) to late protoplasts (distal cytoplasm) corresponds to the characteristics of hyphal apical growth. Autoradiographic studies have shown that in *Saprolegnia* the main site of polysaccharide deposition is located at the first 10 μm of the apex. Beyond 50 μm from the apex, incorporation declines until reaching a low constant level (eight times lower than in the tip zone) (Fèvre & Rougier, 1980, 1982).

Cell wall formation *in vivo* is dependent not only on the localization of glucan synthases but also on the activity of the enzymes converting glucose to the substrate UDPglucose. In *Saprolegnia*, the apical zones must contain these active enzyme systems, as (1 $\rightarrow$ 4)- β -glucan synthase activity observed *in vitro* correlated with the *in vivo* glucose incorporation. On the other hand, distal zones of the hyphae must be poor in enzyme activity producing UDPglucose, as the high (1 $\rightarrow$ 3)- β -glucan synthase activity only led to a low rate of glucose utilization.

In chitinous fungi, active chitin synthase recovered in early protoplasts is located at the hyphal tips, while proteolytically activated enzymes are found in late protoplasts, originating from older parts of the hyphae (Archer, 1977; Isaac *et al.*, 1978).

Our observations might provide a clue as to the mechanisms of control of polysaccharide synthesis in growing hyphae. Cellulose will be produced at the apex, while (1 $\rightarrow$ 3)- β -glucan synthesis will be mainly deposited in subapical zones during later stages of cell wall formation.

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