A novel 1,3-β-glucan synthase from the oomycete Saprolegnia monoica

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No definitive function has been attributed to any one chitin synthase from a filamentous fungus while in Saccharomyces cerevisiae each of the three chitin synthases has a specific role during cytokinesis, mating and septum formation of the budding cells (Cid et al., 1995).

1,3-β-Glucan synthases have been characterized from several filamentous fungi and yeasts (Kang & Cabib, 1986). However, genetic and molecular biological analyses of glucan synthases have not been as comprehensive as the studies on chitin synthases. In yeast, two genes, FKS1 and FKS2, are involved in the formation of 1,3-β-glucan (Ram et al., 1995; El-Sherbeini & Clemas, 1995; Inoue et al., 1995). They encode interchangeable subunits essential for activity of membrane-bound glucan synthases (Mazur et al., 1995). In filamentous fungi, an FKS homologue has been cloned from Aspergillus nidulans and shown to be associated with 1,3-β-glucan synthase activity (Kelly et al., 1996). In the oomycete Saprolegnia monoica, cellulose, 1,3-β-glucans and chitin are cell wall polymers (Bulone et al., 1992). Isolated membrane fractions exhibit a variety of glycosyltransferase activities in vitro. Chitin can be produced from UDP-N-acetylglucosamine (Gay et al., 1993). Two chitin synthase genes have been identified and they are expressed during mycelial growth and proplast regeneration (Mort-Bontemps et al., 1997).

INTRODUCTION

The shape and integrity of fungal cells depend on the cell wall and on the spatial arrangement of the individual polymers. During growth, synthesis and deposition of cell wall components must be highly coordinated to provide and maintain the cell wall architecture (Bartnicki-Garcia, 1990; Wessels, 1993). The main cell wall structural components of fungi are chitin and 1,3-β-glucans. The biochemistry of the enzymes that catalyse the synthesis of these polysaccharides has been extensively studied (Cabib, 1987; Ruiz-Herrera, 1991).

Chitin synthesis in fungi is by far the best characterized enzyme system (Cabib, 1987; Bartnicki-Garcia et al., 1984; Févre et al., 1996). In yeast, chitin biosynthesis is mediated by multiple chitin synthases differing in their pH optima and cation requirements (Choi & Cabib, 1994) and three chitin synthase genes have been characterized (for a review see Bulone, 1993). In filamentous fungi, chitin synthase gene families have also been shown to be large (Din et al., 1996; Mellado et al., 1995).

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1,3-β-Glucan synthases have been characterized from several filamentous fungi and yeasts (Kang & Cabib, 1986). However, genetic and molecular biological analyses of glucan synthases have not been as comprehensive as the studies on chitin synthases. In yeast, two genes, FKS1 and FKS2, are involved in the formation of 1,3-β-glucan (Ram et al., 1995; El-Sherbeini & Clemas, 1995; Inoue et al., 1995). They encode interchangeable subunits essential for activity of membrane-bound glucan synthases (Mazur et al., 1995). In filamentous fungi, an FKS homologue has been cloned from Aspergillus nidulans and shown to be associated with 1,3-β-glucan synthase activity (Kelly et al., 1996). In the oomycete Saprolegnia monoica, cellulose, 1,3-β-glucans and chitin are cell wall polymers (Bulone et al., 1992). Isolated membrane fractions exhibit a variety of glycosyltransferase activities in vitro. Chitin can be produced from UDP-N-acetylglucosamine (Gay et al., 1993). Two chitin synthase genes have been identified and they are expressed during mycelial growth and proplast regeneration (Mort-Bontemps et al., 1997).
Different glucans are produced from UDPglucose depending on the assay conditions: 1,3-β-glucans are synthesized at high substrate concentration while 1,4-β-glucans are formed at low substrate concentration in the presence of Mg²⁺ (Fève & Rougier, 1981; Bulone et al., 1990). The 1,3-β-glucan synthase activities which have been demonstrated in chitinous fungi and oomycetes appear to differ at the level of regulation. Enzymes from chitinous fungi are activated by GTP and ATP while these nucleotides caused hardly any stimulation of the enzymes from the oomycete Achlya ambisexualis (Szaniszlo et al., 1985) and inhibited the enzymes from S. monoica (Fève, 1983).

From all the molecular studies on chitin syntheses and now from those on yeast 1,3-β-glucan synthase, it is becoming evident that a multiplicity of synthases exists in fungi and that the isozymes must have different functions. The objective of this work was to detect and characterize in vitro 1,3-β-glucan synthase activities based on requirements for cofactor or activator, and in relation to the highly polarized organization of the hyphal tip. Polarized growth of the hyphal tip of Saprolegniales has been associated with a gradient in cytosolic free Ca²⁺ from a low Ca²⁺ concentration in subapical regions to a high concentration at the apex (for a review see Jackson & Heath, 1993). It is possible that some enzyme activities are regulated by the cations present at the hyphal apex involved in tip growth. We provide evidence that there are two 1,3-β-glucan synthases in S. monoica; the activity of the novel enzyme is cation-dependent, stimulated by Ca²⁺ and could be involved in apical growth.

**METHODS**

**Fungal strain.** Saprolegnia monoica Pringsheim (no. 53967 Dick, from CBS, Baarn, The Netherlands) was grown for 3 d in Petri dishes (14 cm diam.) containing 150 ml of a modified liquid medium of Machlis (1953) in which the nitrogen source was glutamic acid (10 g l⁻¹).

**Preparation of particulate enzymes and CHAPS solubilization of glycosyltransferases.** The mycelium from liquid cultures was harvested and homogenized in extraction buffer (10 mM Tris/HCl, pH 7.4; 0.5 M sorbitol) with a Virtis homogenizer for 40 s periods at 4°C. Cell walls were discarded after centrifugation at 2000 g for 15 min and the supernatant was centrifuged at 46000 g for 40 min to collect the membranes. The resulting pellet was resuspended in extraction buffer and the protein content was determined by Coomassie blue dye-binding assay using BSA as standard (Bradford, 1976). The final protein concentration was adjusted to 5 mg ml⁻¹ then membrane-bound enzymes were solubilized in 10 mM CHAPS (Sigma) for 30 min at 4°C. After solubilization, the suspension was centrifuged at 50000 g for 60 min and the supernatant was used as the solubilized enzyme preparation.

**β-Glucan synthase assays.** 1,3-β-Glucan synthase activity was assayed in a 300 μl final volume containing 100 μl (i.e. approximately 300 μg protein) of freshly prepared enzyme, 20 mM Tris/maleate buffer, 100 mM cellobiose, 15 mM dithiothreitol, 20 mM UDP[14C]glucose and 0.25 mM UDP[14C]glucose. All assays performed in duplicate were incubated at 25°C for 30 min. Reactions were terminated by addition of 2 ml 95% ethanol and precipitated overnight at −20°C. After addition of powdered cellulose, the reaction products were filtered through glass-fibre filters (Whatman; GF/C). Residues on the filter were successively washed with 2 × 4 ml water and 2 × 4 ml 95% ethanol. Glucose incorporation into ethanol-insoluble glucans was measured by scintillation counting using a PPO/POPPOP toluene scintillation mixture. All the results presented are representative data of triplicate experiments.

**Enzymic hydrolysis of reaction products.** Radioactive glucans produced at pH 5.8 and 8.8 by CHAPS-solubilized enzymes were collected by centrifugation at 13000 g for 15 min. Pellets were resuspended in 200 μl 0.1 M acetate buffer (pH 7.0) containing 50 U α-amylase ml⁻¹ (Fluka) or 200 μl 0.1 M acetate buffer (pH 5.0) containing 0.435 U laminarinase ml⁻¹ (Sigma) or 50 U cellulase ml⁻¹ (Serva). Duplicate pellets were resuspended in 200 μl of the corresponding buffer and used as controls. Mixtures were incubated at 25°C for 4 h. Hydrolysis reactions were terminated by addition of 2 ml cold 5% trichloroacetic acid. The undigested labelled polymers were recovered by filtration as described above and the radioactivity of the remaining polymer was determined.

**Methylation analysis.** Enzyme assays were scaled up to 5 ml using CHAPS-solubilized enzymes. After 1 h incubation, the products were collected as described above, washed thoroughly with water and absolute ethanol and dried under vacuum. The polysaccharide fraction (approx. 87 mg) was methylated by the Hakomori method (Perret et al., 1992), using a minimal amount of reagents. The methylated products were hydrolysed in 90% formic acid for 1 h at 100°C. After evaporation under reduced pressure, a second hydrolysis step was performed with 2 M trifluoroacetic acid for 1 h at 100°C. The acid was evaporated as before and the products were reduced with NaBH₄ and acetylated. The partially methylated alditol acetates were analysed by GLC on a 3% SP 2380 macrobore column (SUPELCO) and GLC-MS analysis was performed on capillary column SP 2380 at 200°C and at an ionizing potential of 70 eV.

**Periodate oxidation.** To the polysaccharide fraction (approx. 250 μg in 1 ml distilled water), myo-inositol hexacetate (60 μg in methanol/water) was added as an internal standard. The mixture was divided into two parts; one for a control and the other for periodate oxidation. To the latter was added 0.1 M sodium metaperiodate (0.75 ml). The reaction was allowed to proceed for 15 h in the dark at 20°C before addition of an excess of ethylene glycol, then dialysed and freeze-dried. Afterwards, control and periodate-treated samples were subjected to 2 M trifluoroacetic acid hydrolysis (100°C, 4 h) and residual amounts of glucose were compared by GLC analysis.

**Sucrose density-gradient centrifugation.** CHAPS-solubilized enzymes (2–3 ml) were layered onto a 30 ml continuous sucrose density gradient (from 15 to 60%, w/w, in 10 mM Tris/HCl buffer, pH 7.4). Centrifugation was carried out for 18 h at 120000 g in a Beckman SW 27 rotor at 4°C. Fractions (2 ml) were collected and assayed for enzyme activities, protein and refractive index.

**Protoplast production.** Mycelium (48 h old) was converted to protoplasts by incubation in 50 ml of a freshly prepared lytic solution (pH 5.8; 0.4 M sodium malate buffer), containing 10 mg driselase ml⁻¹ (Fluka), 1 mg cellulase ml⁻¹ (‘Onozuka R-10’; Serva) and 0.5 M sorbitol, as stabilizer (Gaugy & Fève, 1982). PMSF (30 μl; 120 mM in 95% ethanol) was included in the mixture. At different times of incubation,
RESULTS

Effect of pH on glucan synthase activity

1,3-β-Glucan synthase activity was assayed at high substrate concentration (20 mM UDPglucose), which inhibits 1,4-β-glucan synthesis (Févre & Rougier, 1981), and in Tris/maleate buffer, which allows measurement of the activity at pH values ranging from 5.0 to 9.0. Using membrane-bound enzymes, the optimum activity was obtained at pH 5.8, but there was a second peak at pH 8.3, this alkaline activity representing about 50% of the activity of the main peak (Fig. 1). Using CHAPS-solubilized enzymes, a similar profile of pH-dependent enzyme activity was obtained but the activity detected at pH 8.3 was higher, corresponding to about 60% of the maximal acid activity (about 90% at pH 8.8). This relative increase in activity may correspond to the unmasking or release of enzymes enclosed in membranes. Two peaks of enzyme activity were detected previously (Févre & Dumas, 1977) when enzymes were extracted in buffer containing 1 mM EDTA. However, the alkaline activity was very low, representing less than 20% of the maximal peak. In the experiments described above, enzymes were prepared in the absence of EDTA and their activities were higher. This indicates that the enzyme with an alkaline pH optimum probably requires cations for optimal activity while these cations are not needed for the enzyme with an acid pH optimum. We name the enzyme activity with acid pH optimum 1,3-β-glucan synthase 1 (1,3-β-GS1) and the enzyme activity with alkaline pH optimum 1,3-β-glucan synthase 2 (1,3-β-GS2).

![Fig. 1. Influence of pH on β-glucan synthesis by the particulate fraction (■) and CHAPS-solubilized enzymes (□) from S. monoica. One hundred per cent activity corresponds to 17.6 and 52.8 nmol glucose incorporated min⁻¹ (mg protein)⁻¹ by the particulate and solubilized enzymes, respectively.](image)

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Activity (%) at pH 5.8</th>
<th>Activity (%) at pH 8.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>46.1</td>
<td>160.8</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>29.6</td>
<td>167.9</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>68.9</td>
<td>181.8</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>100.1</td>
<td>78.8</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>61.9</td>
<td>58.7</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>25.9</td>
<td>20.2</td>
</tr>
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</table>

One hundred per cent activity corresponds to 17.6 nmol glucose incorporated min⁻¹ (mg protein)⁻¹ at pH 5.8 (1,3-β-GS1) and 52.8 nmol glucose incorporated min⁻¹ (mg protein)⁻¹ at pH 8.8 (1,3-β-GS2). Results are representative data of four experiments.

Effects of divalent cations on the enzyme activities

Various cations used as chloride salts were tested at a concentration of 2 mM and the enzyme activity was assayed at pH 5.8 and 8.8. Ca²⁺, Mn²⁺ and Mg²⁺ stimulated the alkaline activity to a similar level while they inhibited the acid activity; Mn²⁺ produced the highest inhibition, i.e. 70% (Table 1). Co²⁺ and Zn²⁺ inhibited both enzyme activities. Ni²⁺ reduced only the alkaline activity. These results show that the 1,3-β-GS2 activity, in contrast to the 1,3-β-GS1 activity, is cation-dependent. This was confirmed by the action of EDTA, which inhibited 1,3-β-GS2 activity by probably chelating the cations present in the enzyme preparation. Higher concentrations of Ca²⁺, Mn²⁺ and Mg²⁺ produced an increasing inhibition of the activity assayed at pH 5.8 and its complete inhibition at 10 mM. When the enzymes were assayed at pH 8.8, these cations had a maximal stimulation at 2 mM. Higher concentrations of Mn²⁺ reduced the stimulatory effect while the stimulation remained at the same level up to 10 mM Mg²⁺ or Ca²⁺ (data not shown). Similar results were obtained when 1,3-β-glucan synthase activities were assayed at pH values varying from 5.5 to 9.0. Mn²⁺ (6 mM) completely inhibited the acid enzyme activity while the alkaline activity (from pH 7.5) was stimulated (Fig. 2). Similar results were obtained using Ca²⁺ or Mg²⁺ (data not shown). The requirement for cations for stimulation of the alkaline activity, but not for the acid enzyme, was confirmed by performing enzyme assays in the presence of 1,3-β-glucan synthase from Saprolegnia monoica.
Influence of Mn\(_2^+\) on \(\beta\)-glucan synthesis by CHAPS-solubilized enzymes from \(S.\) monoica assayed at different pH values. Enzyme activity was assayed as described in Methods and 50 \(\mu\)l of a stock Mn\(^{2+}\) solution was added to the assay mixtures to obtain a 6 mM final concentration. Enzyme activity is expressed as nmol glucose incorporated min\(^{-1}\). •, Assays in the presence of 6 mM Mn\(^{2+}\); ○, assays in the absence of Mn\(^{2+}\).

Influence of EDTA on \(\beta\)-glucan synthesis by CHAPS-solubilized enzymes from \(S.\) monoica at different pH values. Enzyme activity was assayed as described in Methods and 50 \(\mu\)l of a stock EDTA solution was added to the assay mixtures to obtain an 8 mM final EDTA concentration. Enzyme activity is expressed as nmol glucose incorporated min\(^{-1}\). •, Assays in the presence of 8 mM EDTA; ○, assays in the absence of EDTA.

Chymotrypsin stimulation of the alkaline 1,3-\(\beta\)-glucan synthase activity

Addition (from 1 to 10 U) of TPCK-treated trypsin to the enzyme assay had no effect on 1,3-\(\beta\)-GS1 while 1,3-\(\beta\)-GS2 activity was reduced by about 20% at the highest protease concentration used (data not shown). TLCK-treated chymotrypsin caused approximately 20% diminution of 1,3-\(\beta\)-GS1 activity. The alkaline activity increased by approximately 50% in the presence of 1 U protease but increasing amounts of the protease (up to 10 U) progressively reduced the stimulatory effect (Fig. 4).

Nucleotide triphosphates inhibit both 1,3-\(\beta\)-glucan synthase activities

GTP and ATP are efficient effectors of fungal 1,3-\(\beta\)-glucan synthase (Mazur & Baginsky, 1996; Szaniszlo et al., 1985) while they both inhibit 1,3-\(\beta\)-glucan synthase activities in \(S.\) monoica. These nucleotides were included in assays at final concentrations varying from 0.2 to 600 \(\mu\)M and produced a diminution of glucose incorporation at both optimal pH values. At 20 \(\mu\)M, these nucleotides inhibited the acid and alkaline activities by about 10% and 40%, respectively; about 50% inhibition of each activity was obtained at the highest concentration tested.

Kinetics of glucan synthesis

CHAPS-solubilized enzymes were assayed in the presence of increasing UDPglucose concentrations: 1,3-\(\beta\)-GS1 was assayed at pH 5.8 in the presence of 8 mM EDTA to inhibit the enzyme with alkaline pH optimum and 1,3-\(\beta\)-GS2 was assayed at pH 8.8 in the presence of 6 mM Mn\(^{2+}\) to inhibit the enzyme with acid pH optimum (Fig. 5). Using these conditions, synthesis of glucan from UDPglucose followed simple Michaelis–
A novel 1,3-\(\beta\)-glucan synthase from *Saprolegnia*

**Fig. 5.** Kinetics of CHAPS-solubilized \(\beta\)-glucan synthases from *S. monoica* assayed at pH 5.8 (○) and 8.8 (●). Results are representative data of triplicate experiments.

**Table 2.** Glucan synthase activities of cell-free extracts of protoplasts from *S. monoica*

Protoplasts were released after 45 and 90 min incubation with lytic solution containing driselase, cellulase and 0.5 M sorbitol in 0.4 M sodium maleate buffer (pH 5.8) (for further details see Methods). CHAPS-solubilized enzymes and detergent-treated membranes were assayed at pH 5.8 and 8.8. Activities are expressed as nmol glucose incorporated min\(^{-1}\) (mg protein\(^{-1}\)). Results are representative data of triplicate experiments.

<table>
<thead>
<tr>
<th>Enzyme activities in protoplasts released after:</th>
<th>45 min lysis</th>
<th>90 min lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3-(\beta)-GS2 CHAPS-solubilized enzymes</td>
<td>49.22</td>
<td>21.42</td>
</tr>
<tr>
<td>Detergent-treated membranes</td>
<td>1.56</td>
<td>0.86</td>
</tr>
<tr>
<td>1,3-(\beta)-GS1 CHAPS-solubilized enzymes</td>
<td>0.23</td>
<td>0.08</td>
</tr>
<tr>
<td>Detergent-treated membranes</td>
<td>0.28</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Menten kinetics. The \(K_m\) and \(V_{max}\) values with respect to UDPglucose were 1.25 mM and 4.84 nmol glucose incorporated min\(^{-1}\), respectively, for 1,3-\(\beta\)-GS1, and 0.63 mM and 2.21 nmol glucose incorporated min\(^{-1}\), respectively, for 1,3-\(\beta\)-GS2.

**Glucan synthase activities in protoplasts**

The digestion of a fungal colony by lytic enzymes enables a sequential fractionation of mycelium as protoplast production has been considered as a sequential release of cytoplasm from apical to subapical parts (Isaac *et al.*, 1978; Girard & Fèvre, 1984). Protoplasts were collected after 45 and 90 min lysis, washed in the osmotic buffer, homogenized, and membranes were collected then CHAPS-solubilized. After centrifugation at 50000 g for 60 min, solubilized enzymes were separated from the CHAPS-treated membranes and both fractions were used for the assay of 1,3-\(\beta\)-glucan synthase activities (Table 2). Most of the enzyme activities were solubilized except for the enzyme with acid pH optimum of the late protoplasts, which remained mainly associated with the detergent-treated membranes. Using CHAPS-solubilized enzymes, the activity of the enzyme with alkaline pH optimum, recovered in the protoplasts released after 45 min lysis, was 2.3 times higher than the activity detected in late protoplasts. In both fractions, 1,3-\(\beta\)-GS2 activity was much higher than the 1,3-\(\beta\)-GS1 activity; the latter activity was very low compared to that detected in mycelial extracts by Girard & Fèvre (1984). These results suggest that 1,3-\(\beta\)-GS2 may be localized in the apical part of the hypha. It is therefore possible that 1,3-\(\beta\)-GS1 and 1,3-\(\beta\)-GS2 are found at different sites in the hypha since the previously described glucan synthase (i.e. 1,3-\(\beta\)-GS1) was found associated with subapical cytoplasm (Girard & Fèvre, 1984).

**Sucrose density-gradient centrifugation of solubilized enzymes**

Membrane-bound glucan synthases were CHAPS-solubilized then centrifuged on a sucrose density gradient. After 18 h centrifugation (isopycnic centrifugation), the enzyme activities were clearly separated from the bulk of solubilized proteins which did not enter the gradient. The two enzyme activities were repeatedly separated and the maximal activity of 1,3-\(\beta\)-GS2 and 1,3-\(\beta\)-GS1...
Fig. 7. Characterization of the 1,3-β-glucan, synthesized by 1,3-β-GS2 at pH 8.8, by methylation analysis. (a) GLC of the partially methylated alditol acetates was performed on a 3% SP 2380 macrobore column for 30 min. Two partially methylated glucose derivatives were identified and the major methylated sugar was eluted at the same retention time as 2,4,6-tri-O-methylglucose (11 min). The arrow indicates the elution position for 2,4,6-tri-O-methylglucose. (b) GLC-MS analysis was performed on capillary column SP 2380 at 200 °C and at an ionizing potential of 70 eV. The peak that eluted at a retention time of 11 min shows the typical mass spectrum for a 2,4,6-tri-O-methylglucose. No di-O-methylglucose derivative was detected, indicating the unbranched nature of the synthesized glucan.

Table 3. Hydrolysis of glucan synthesized by CHAPS-solubilized enzyme preparations from S. monoica

<table>
<thead>
<tr>
<th>Lytic enzyme</th>
<th>Residual radioactive glucans (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,3-β-GS1</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>a-Amylase</td>
<td>83</td>
</tr>
<tr>
<td>Cellulase</td>
<td>91</td>
</tr>
<tr>
<td>Laminarinase</td>
<td>14</td>
</tr>
</tbody>
</table>

Polymers produced under optimized acid and alkaline assay conditions were subjected to the action of purified hydrolases (Table 3). The radioactive products were almost totally hydrolysed by laminarinase (i.e. 1,3-β-glucanase) as approximately 90% of the radioactivity from the polymer was released into the supernatant. Cellulase and amylase released only approximately 10% of the radioactivity. This indicates that 1,3-β-linked polymers were the major products synthesized under both assay conditions. These results are in agreement with our previous findings which indicated that the enzyme with acid pH optimum synthesized 1,3-β-glucans at high substrate concentration in the absence of cation (Bulone et al., 1992). They also indicate that the same type of polymers can be produced by the enzyme with alkaline pH optimum.

The demonstration of the 1,3-β-linked glucan structure of the products synthesized by 1,3-β-GS2 at pH 8.8 has been done by methylation analysis and periodate oxidation. The products synthesized by 1,3-β-GS2 were methylated, hydrolysed and then analysed by GLC as alditol acetates and the peak identification was confirmed by GLC-MS analysis. Only two partially methylated glucose derivatives were identified and the main methylated sugar was eluted at the same retention time as 2,4,6-tri-O-methylglucose (Fig. 7a). The other methylated sugar, present in very small amounts and with a retention time of 15 min, was identified as 2,3,4,6-tetra-O-methylglucose, corresponding to the nonreducing end.
of the glucan. GLC-MS analysis of methylated reaction products showed typical fragments of 2,4,6-tri-O-methylglucose derivatives (Fig. 7b). No di-O-methylglucose derivative could be identified, therefore indicating the unbranched nature of the synthesized glucan. Because of the very small amount of the tetra-O-methylglucose detected, it can be inferred that the glucan had a chain length higher than degree of polymerization 20. This structure was confirmed by periodate oxidation. Quantitative analysis was performed by solubilizing the products in the presence of inositol hexacetate used as an internal standard protected from oxidation by periodic acid. An aliquot was kept for reference and the rest of the mixture was subjected to periodate oxidation. After hydrolysis, followed by GLC analysis, both the reference and the periodate-treated sample yielded the same amount of glucose and the total recovery of glucose showed that no significant oxidative degradation of the glucan had occurred (data not shown), proving its 1,3-glucan structure. Therefore, the results of structural analysis, considered in conjunction with the susceptibility of the glucan to laminarinase hydrolysis, clearly demonstrate that the synthesized polymer consisted of a 1,3-β-linked linear glucan.

**DISCUSSION**

The results suggest that *S. monoica* has two 1,3-β-glucan synthase activities, one of which is apparently novel. Taking into consideration the differences in pH optimum and the effect of divalent ions, each enzyme activity could be specifically assayed in the presence of the other. The novel enzyme is active at alkaline pH and displays properties that distinguish it from the 1,3-β-glucan synthase previously described (Févre & Rougier, 1981; Bulone et al., 1990). The enzyme with alkaline pH optimum is stimulated by the divalent cations Ca²⁺, Mn²⁺ and Mg²⁺. Its activity was higher in protoplasts released after 45 min than in protoplasts collected after 90 min lysis, suggesting that the enzyme may be located in the apical part of the hypha. This contrasts strongly with the Ca²⁺, Mn²⁺ and Mg²⁺ inhibition of the former characterized enzyme and with its subapical localization (Girard & Févre, 1984). A concentration gradient of free Ca²⁺ occurs in the cytosol of growing hyphae with the concentration of the divalent cation highest at the apex (Jackson & Heath, 1993; Levina et al., 1995). The fact that a Ca²⁺ gradient is always present in growing hyphae and is absent from nongrowing hyphae indicates an essential role for Ca²⁺ in tip growth. The sensitivity of the 1,3-β-glucan synthase activities to divalent cations suggests that Ca²⁺ and other cations may play a regulatory role in cell wall polymer biosynthesis during hypha elongation. It is interesting to note that 1,4-β-glucan synthase and chitin synthase activities of *S. monoica* are also cation-dependent (Févre & Rougier, 1981; Bulone et al., 1992; Gay et al., 1993). It is possible that the role played by Ca²⁺ in organizing the extreme polarization of hyphae and controlling tip growth could be extended to the regulation of the enzyme activities responsible for the biosynthesis of cell wall poly-

1,3-β-Glucan synthases are membranous and can be solubilized by CHAPS and other detergents such as digitonin or octylglucoside (L. Gay and others, unpublished results). The enzymes have been solubilized and separated as distinct peaks in density gradients, suggesting that they consist of different protein complexes. We do not know if these complexes are composed of different regulatory subunits associated with the same catalytic subunit or if they correspond to entirely different multiprotein complexes. *Sac. cerevisiae* contains two genes encoding proteins associated with 1,3-β-glucan synthase activity (Ram et al., 1995; El-Sherbeini & Clemons, 1995; Inoue et al., 1995) and so it is possible that the enzyme activities revealed in *S. monoica* may be supported by two different complexes. This would not be surprising as chitin synthase activity in *S. monoica* appears to be encoded by a multigene family similar to that detected in chitinous fungi (Mort-Bontemps et al., 1997). Characterization of reaction products showed that both β-glucan synthase activities synthesize linear 1,3-β-glucans. No branch points at Cs and no 1,4-β-linked oligosaccharides were detected in the products synthesized in vitro although they have been found in glucans from the mycelial cell walls of oomycetes (Bartnicki-Garcia, 1970). This implies that other enzymes, i.e. glycosyltransferases and glucan synthases, must exist but were not detected due to inappropriate assay conditions or inactivation during extraction procedures.

The 1,3-β-glucan synthases from *S. monoica* were inhibited by GTP (or ATP) and GTP is required for maximal activity of 1,3-β-glucan synthases from chitinous fungi (Szaniawski et al., 1985). Furthermore, we did not detect cross-hybridization to the yeast FK5 gene in Southern blots of genomic DNA (M. Mort-Bontemps, unpublished results). These differences may have a phylogenetic basis as oomycetes and chitinous fungi constitute widely differing groups (Cavaliere-Smith, 1987). The novel 1,3-β-glucan synthase we have detected resembles plant enzymes requiring divalent cations but not GTP for activation. It will be of interest to know if β-glucosides identified as natural effectors of plant enzymes (Ohana et al., 1991; Ng et al., 1996) stimulate the oomycete enzymes. Although no 1,3-β-glucan synthase has yet been purified or completely characterized, preliminary data on the effects of cofactors or activators suggest that distinct families of 1,3-β-glucan synthases may be found for each of plants, oomycetes and chitinous fungi.

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