1,3- β -Glucanase, 1,6- β -Glucanase and β -Glucosidase Activities of *Sclerotium glaucum*: Synthesis and Properties

By PETER RAPP

Institut für Biochemie und Biotechnologie der Technischen Universität Braunschweig,
D-3300 Braunschweig, FRG

(Received 19 April 1989; revised 21 July 1989; accepted 4 August 1989)

The filamentous fungus *Sclerotium glaucum* excreted significant amounts of 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities when the culture medium was depleted of carbon sources. During starvation small amounts of intracellular 1,3- β - and 1,6- β -glucanase and β -glucosidase activities were also detected. Very low levels of β -glucanase activity remained bound to mycelium and some activity was found loosely attached to the cells and/or to water-soluble 1,3- β -/1,6- β -glucan adhering to the cell walls. During active growth intracellular 1,3- β -glucanase and mycelium-bound 1,3- β - and 1,6- β -glucanase activities were detected in small or trace amounts. During hyphal growth very low levels of 1,3- β - and 1,6- β -glucanase activities were also found to be weakly associated with the cells and/or with water-soluble β -glucan covering the hyphae. Cycloheximide inhibited the increase in intra- and extracellular 1,3- β - and 1,6- β -glucanase and β -glucosidase activities. This indicated that *de novo* protein synthesis was involved in the intra- and extracellular appearance of these three enzyme activities in derepressed cells. The formation of the extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities was regulated by catabolite repression. 1,3- β - and 1,6- β -glucanase activities were uncompetitively inhibited and β -glucosidase activity noncompetitively inhibited by glucose and glucono- δ -lactone. Optimum pH and temperature values as well as thermal stabilities of the three extracellular enzyme activities were determined. Almost all of the β -glucosidase activity but only one-third of the extracellular 1,3- β - and 1,6- β -glucanase activities were found to bind to Con A-Sepharose. Under conditions of carbon limitation almost 90% of the extracellular 1,3- β -/1,6- β -glucan excreted during fungal growth was degraded by the extracellular β -glucanases.

INTRODUCTION

Many species of filamentous fungi produce enzymes capable of hydrolysing 1,3- β - and 1,6- β -linked glucans (Reese & Mandels, 1959; Reese *et al.*, 1962; Chesters & Bull, 1963). They have been classified as exo-1,3- β -glucanase (1,3- β -D-glucan glucohydrolase; EC 3.2.1.58), endo-1,3- β -glucanase (1,3- β -D-glucan glucanohydrolase; EC 3.2.1.6), endo-1,6- β -glucanase (1,6- β -D-glucan glucanohydrolase; EC 3.2.1.75) and β -glucosidase (β -D-glucoside glucohydrolase; EC 3.2.1.21). Endoglucanases attack a glucan chain more or less randomly, while exoglucanases are able to cleave glucose from its non-reducing end. Furthermore, endoglucanases and β -glucosidases act in such a way that the products retain the configuration of the substrates, whereas exoglucanases invert the configuration of the products (Reese, 1977). Exoglucanases and β -glucosidases differ also in other respects such as bond specificity, substrate affinity, inhibition and glucosyltransferase activity (Reese, 1977). Of note is the finding that the 1,6- β -glucanases act only as endoenzymes (Reese *et al.*, 1962).

Abbreviations: DNS, 3,5-dinitrosalicylic acid; pNPG, *p*-nitrophenol β -glucopyranoside.

Among the most frequently occurring polysaccharides of fungal cell walls are 1,3- β -glucans often linked with 1,6- β -glucosyl residues or 1,6- β -oligosaccharyl moieties (Catley, 1983). Numerous fungi, including *Sclerotium glaucanicum* (Halleck, 1967; Johnson *et al.*, 1963), *Sclerotium rolfii* (Halleck, 1967; Rinaudo & Vincendon, 1982), *Helotium* sp. and *Plectania occidentalis* (Davis *et al.*, 1965), *Schizophyllum commune* (Kikumoto *et al.*, 1970, 1971; Niederpruem *et al.*, 1977) and *Monilinia fructicola* (Nachtwey, 1989) excrete water soluble 1,3- β -linked D-glucans with branches of single D-glucopyranosyl groups 1,6- β -linked to every third residue of the main chain. Conformational studies of this polysaccharide, which has been termed scleroglucan, indicate that the 1,3- β -linked chains are arranged in a triple helix (Yanaki *et al.*, 1981; Bluhm *et al.*, 1982). Singh *et al.* (1974) demonstrated that the extracellular 1,3- β -/1,6- β -glucan from *S. glaucanicum* has strong antitumoral activity. In some carbon-limited fungal cultures, mycelium and extracellular 1,3- β -/1,6- β -glucans are hydrolysed by β -glucanases (Catley, 1983). 1,3- β - and 1,6- β -glucan degrading enzymes are also associated with morphogenesis (Fèvre, 1979; Wessels & Sietsma, 1979; Kamada *et al.*, 1982, 1985). The present study was undertaken to examine the formation, location and regulation of 1,3- β - and 1,6- β -glucan degrading enzymes from *S. glaucanicum*.

METHODS

Chemicals. Laminarin was purchased from Sigma. Pustulan was obtained from Calbiochem and *p*-nitrophenyl β -glucopyranoside (*p*NPG) from Merck. Periodate-oxidized laminarin was prepared according to Goldstein *et al.* (1965). All other reagents were analytical grade from commercial sources.

Organism and cultivation conditions. *Sclerotium glaucanicum* (NRRL 3006; obtained from CBS, Baarn, The Netherlands) was maintained on Sabouraud dextrose agar (Difco) at 4 °C and subcultured every 8 weeks by incubation at 27 °C for 4 to 5 d. The agar slant was inoculated with a mycelial disc (about 5 mm diam.).

Submerged cultures were grown in a basal medium consisting of (per litre of deionized water): NaNO₃, 3.0 g; K₂HPO₄·3H₂O, 1.3 g; KCl, 0.5 g; MgSO₄·7H₂O, 0.5 g; FeSO₄·7H₂O, 0.05 g; citric acid, 1H₂O, 0.7 g; yeast extract, 1.0 g. The pH before autoclaving was adjusted to 4.5. Seed cultures were grown by inoculating a mycelial disc (about 5 mm diam.) into a 500 ml shake-flask containing 100 ml of basal medium plus 1% (w/v) carbon source on a rotary shaker (100 r.p.m.) at 27 °C for 3 to 4 d. A 5 ml sample of this culture was used to inoculate 250 ml of basal medium plus 1% (w/v) carbon source in a 1 litre flask and cultivated with shaking as before.

Growth measurement. Fungal growth was followed by determining the mycelial dry weight. Mycelium was separated from polysaccharide by centrifugation at 15000 *g*. The pellet was washed twice with distilled water and dried to a constant weight under reduced pressure at 40 °C.

Determination of polysaccharide content. Polysaccharide was precipitated from the culture supernatant by adding 2 vols of propan-2-ol, washed with 70% (w/v) propan-2-ol and dried to constant weight under reduced pressure at 40 °C.

Isolation and purification of polysaccharide. Extracellular 1,3- β -/1,6- β -glucan was isolated and purified according to Rapp *et al.* (1979).

Analysis of glucose and protein. Glucose concentration was measured with a Glucose Analyzer (Yellow Spring Instruments). The determination is based on the oxidation of glucose to glucono- δ -lactone and hydrogen peroxide by glucose oxidase immobilized in a membrane. Hydrogen peroxide is oxidized at a platinum anode and the current flow between this anode and a silver cathode is linearly proportional to the concentration of glucose. Soluble protein was measured by the Lowry method using bovine serum albumin as the standard.

Enzyme assays. 1,3- β -Glucanase activity was assayed by incubating 1 ml 0.5% (w/v) laminarin in 0.07 M-sodium phosphate buffer, pH 4.0 with 1 ml of enzyme solution at 55 °C for 5 min. The reaction was stopped in an ice-bath; 3 ml of 3,5-dinitrosalicylic acid (DNS) reagent (Bruner, 1964) was then added and the mixture heated in a boiling water-bath for 12 min. As a control, 1 ml of laminarin solution was incubated and cooled, and 1 ml of enzyme solution and 3 ml of DNS reagent were added to correct for the reducing sugars in the substrate and the enzyme solution. Reducing sugar equivalents were measured in both the original and the control solutions by the colorimetric method of Miller *et al.* (1960) with glucose as the standard. 1,6- β -Glucanase activity was assayed as described above except that pustulan was used as the substrate. Activity against periodate-oxidized laminarin was assayed as described above except that 0.5% (w/v) periodate-oxidized laminarin in 0.07 M-sodium phosphate buffer, pH 4.5, was used as the substrate. One unit (U) of β -glucanase activity was defined as the amount of enzyme that produced 1 μ mol of reducing sugars min⁻¹ under the given conditions.

β -Glucosidase activity was determined with *p*NPG as the substrate. The reaction mixture contained 0.5 ml 0.05 M-*p*NPG in 0.07 M-sodium phosphate buffer, pH 5.0, and 0.5 ml of enzyme solution. After incubation at 40 °C for 10 min 1 ml 1 M-sodium carbonate was added and the mixture was chilled to about 4 °C. The absorbance

was measured at 400 nm. A blank was always used with water instead of enzyme solution. β -Glucosidase activity was defined and calculated as described previously (Stoppok *et al.*, 1982).

Preparation of cell-free extracts for the assay of intracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activity. Several methods were applied to prepare cell-free extracts from *S. glucanicum* (Kottutz, 1989). Most effective was the disruption of mycelium with a Bachofen Dyno-mill (beads 0.5 to 0.75 mm diam.), a Manton Gaulin high pressure homogenizer or a Waring blender. The latter method was chosen since it allowed the use of small volumes. Shake-flask cultures (25 ml) grown in basal medium with 1% (w/v) glucose were centrifuged for 30 min at 15000 g at 4 °C. The pelleted mycelium was washed twice with distilled water, then suspended in 20 ml of distilled water and disrupted in a Waring blender (maximum output) for 10 min with cooling. The crude extract was centrifuged again as described above. Samples were examined microscopically to confirm that most of the hyphae had been disrupted; the cell-free extracts were then assayed for 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities.

Determination of cell-bound 1,3- β - and 1,6- β -glucanase activity. A culture (100 ml) of *S. glucanicum*, grown as described above (carbon source: 1%, w/v, glucose) was centrifuged at 15000 g at 4 °C. The mycelium was washed twice with distilled water and suspended in 5 ml 0.07 M-sodium phosphate buffer, pH 4.0. A 1 ml sample of this cell suspension and 1 ml 0.5% (w/v) laminarin or pustulan, in 0.07 M-sodium phosphate buffer, pH 4.0, were incubated at 55 °C for 5 min and the enzyme activities measured as described above. As a control, 1 ml of substrate solution was incubated and cooled and 1 ml of cell suspension and 3 ml of DNS reagent were added to correct for reducing sugars in the substrate and in the cell suspension.

Determination of 1,3- β - and 1,6- β -glucanase activity released by washing of the mycelium. Both β -glucanase activities were assayed in the enzyme solution obtained by washing the mycelium twice with distilled water.

Binding to Con A-Sepharose. Crude enzyme solution with a pH value of about 6.0 and a protein content of 10 to 50 mg was applied to a Con A-Sepharose column (2 $\times$ 11 cm), previously equilibrated with 0.1 M-sodium acetate buffer, pH 6.0, containing 1 M-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂ and 1 mM-MnCl₂. Unbound substances were eluted with 10 column vols of the same buffer and assayed for 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activity. As control 10 column vols of the buffer solution were added to the same volume of crude enzyme solution, which was applied to the column and also assayed for both β -glucanase and β -glucosidase activities.

1,3- β -/1,6- β -Glucan hydrolysis by crude enzyme solutions containing 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activity. Purified extracellular 1,3- β -/1,6- β -glucan (0.54 g) was dissolved in 100 ml 0.07 M-sodium phosphate buffer, pH 4.5, by heating at 121 °C for 20 min. Supernatant (100 ml) from a shake-flask culture (250 ml culture in a 1 litre shake-flask; 100 r.p.m.) with an initial concentration of 1% (w/v) glucose and grown for 240 and 264 h was added aseptically and the resultant solutions were incubated on a rotary shaker (100 r.p.m.) at 27 °C. Samples (20 ml) were taken aseptically at intervals and heated for 10 min in a boiling water bath. After cooling the concentrations of polysaccharide, glucose and reducing sugar equivalents were measured. The latter was assayed by the 3,5-DNS method of Brunner (1964). For controls 100 ml of heat inactivated culture supernatant was added to 100 ml of the β -glucan solution and incubated at 27 °C under shaking (100 r.p.m.). Samples were taken and assayed as described above.

RESULTS

Production and localization of 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities

Growth and production of extracellular 1,3- β -/1,6- β -glucan during cultivation in shake-flasks with an initial glucose concentration of 1% (w/v) are shown in Fig. 1. After 72 h cultivation, when the concentration of glucose had fallen to about 0.03% (w/v), the amount of mycelium and extracellular polysaccharide decreased (Fig. 1) and low 1,3- β - and 1,6- β -glucanase as well as β -glucosidase activities could be measured in the culture supernatant (Fig. 2). These activities increased during continued starvation while mycelium and extracellular β -glucan were degraded further (Fig. 1). Extracellular 1,6- β -glucanase and β -glucosidase activities reached maximum values after about 170 h cultivation (Fig. 2). Highest extracellular 1,3- β -glucanase activity was reached even later after about 220 h cultivation (Fig. 2). 1,3- β -Glucanase activity was consistently higher than the other extracellular enzyme activities assayed. Low levels of intracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities were also measured during cultivation (Fig. 3). Comparing Fig. 2 with Fig. 3 it is evident that intracellular enzyme activities reached maximum values about 20 h earlier than the corresponding extracellular enzyme activities. In contrast to extracellular enzyme activities, which slowly decreased during prolonged incubation, intracellular activities rapidly declined after having reached their maxima (Figs 2 and 3). These two observations and the low maximum intracellular enzyme

Fig. 1. Growth and production of extracellular 1,3- β -/1,6- β -glucan during cultivation in shake-flasks (250 ml per 1 litre flask; 100 r.p.m.) with an initial glucose concentration of 1% (w/v). After 48 h growth 220 μ g cycloheximide ml^{-1} was added (arrowed). $\circ$, Mycelial dry wt; $\bullet$, mycelial dry wt after addition of cycloheximide; $\square$, extracellular 1,3- β -/1,6- β -glucan; ∇ , residual glucose in culture supernatant; $\blacktriangledown$, residual glucose in culture supernatant after addition of cycloheximide. Values are means of two independent experiments.

Fig. 2

Fig. 3

Fig. 2. Production of extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities during cultivation in shake-flasks (250 ml per 1 litre flask; 100 r.p.m.) with an initial glucose concentration of 1% (w/v). After 48 and 124 h growth 220 μ g cycloheximide ml^{-1} was added (arrowed). $\triangle$, 1,3- β -Glucanase activity; $\square$, 1,6- β -glucanase activity; ∇ , β -glucosidase activity. $\blacktriangle$, $\blacksquare$, $\blacktriangledown$, Corresponding activities after addition of cycloheximide. Values are means of two independent experiments.

Fig. 3. Production of intracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities during cultivation in shake flasks (250 ml per 1 litre flask; 100 r.p.m.) with an initial glucose concentration of 1% (w/v). After 48 and 124 h growth 220 μ g cycloheximide ml^{-1} was added (arrowed). $\triangle$, 1,3- β -Glucanase activity; $\square$, 1,6- β -glucanase activity; ∇ , β -glucosidase activity. $\blacktriangle$, $\blacksquare$, $\blacktriangledown$, Corresponding activities after addition of cycloheximide. Values are means of two independent experiments.

Table 1. *1,3- β - and 1,6- β -glucanase activities bound to mycelium, released by washing and present in the supernatant*

Cultures were grown in shake-flasks (100 ml culture per 500 ml flask; 100 r.p.m.) with an initial glucose concentration of 1% (w/v). The inoculum was a mycelial disc (about 5 mm diam.) from an agar slant. Values given are means of two independent experiments.

	Time (h)	1,3- β -Glucanase (U ml ⁻¹)			1,6- β -Glucanase (U ml ⁻¹)		
		72	98.5	128	72	98.5	128
Glucose concn in culture	...						
supernatant (mg ml ⁻¹)	...	7.06	4.06	0.16	7.06	4.06	0.16
Mycelium-bound enzyme activity*		0.002	0.009	0.007	0.003	0.002	0.003
Enzyme activity released by repeated washing with water		0.002	0.019	0.026	0.006	0.016	0.016
Enzyme activity in culture supernatant		0.0	0.0	0.140	0.0	0.0	0.100

* Mycelium-bound activity was measured after repeated washing of mycelium with water. The total contents of one flask were used for determinations.

activities suggest that the three enzyme activities were exported. During growth, very low intracellular 1,3- β -glucanase (Fig. 3) and cell-bound 1,3- β - and 1,6- β -glucanase activities were measured (Table 1). Moreover, during fungal growth low levels of β -glucanases were also found loosely attached to the mycelium and/or to water-soluble β -glucan surrounding the cells. Only when the glucose concentration fell far below 4 mg ml⁻¹ were 1,3- β -glucanase and 1,6- β -glucanase activities detected in the culture supernatant (Table 1). During secretion of the enzymes into the culture medium, a small portion of enzyme activity remained bound to mycelium and a larger portion was weakly attached to the cells and/or to water-soluble β -glucan covering the mycelium, which could be removed by washing with water (Table 1).

Some cultures were inoculated with a mycelial disc (about 5 mm diam.) from an agar slant instead of 2 ml of a shake-flask culture as used above. Growth and β -glucanase production were delayed (Table 1), and at the beginning of incubation no β -glucanase activity could be detected in the culture.

Cycloheximide was added to shake-flask cultures after 124 h cultivation, when autolysis of the fungus and degradation of extracellular 1,3- β -/1,6- β -glucan could clearly be detected. After addition of cycloheximide no further increase in intra- and extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities could be measured (Figs 2 and 3). In the absence of cycloheximide 86% of the extracellular β -glucan and 75% of mycelium produced during cultivation in shake-flasks were degraded within 140 and 170 h, respectively. After 210 h cultivation 0.75 mg extracellular polysaccharide ml⁻¹ was left; the amount did not decrease thereafter (Fig. 1).

Repression of extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities

During growth of *S. glucanicum* in the presence of an excess of carbon source, no extracellular β -glucanase and β -glucosidase activities could be measured (Figs 1 and 2; Table 1). After the carbon source had reached a low concentration, 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activity appeared in the culture supernatant and increased significantly thereafter (Figs 1, 2, 4, 5 and 6; Table 1). Addition of an excess of glucose or another readily utilizable carbon source caused production of these enzyme activities to be repressed again. In the experiments described, xylose was used as the repressing carbon source, since glucose was found to inhibit 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities (Table 2). It is apparent from Figs 4 to 6 that addition of 0.133 M-xylose (2%, w/v) repressed the formation of the three extracellular enzyme activities, whereas 0.066 M-xylose only delayed (Figs 4 and 5) or lessened (Fig. 6) the increase in enzyme activities. During repression by xylose hyphal growth occurred again (Figs 4 to 6). In those experiments where most of the xylose was consumed and the repression relieved, overall enzyme synthesis exceeded that of the control due to the additional cell mass produced during repression.

Fig. 4. Repression of extracellular 1,3- β -glucanase activity by xylose. *S. glaucanicum* was grown in shake-flasks (250 ml per 1 litre flask; 100 r.p.m.) with an initial concentration of 1% (w/v) glucose. After 144 h 0.133 M and after 229 h 0.066 M and 0.133 M-xylose was added (arrowed). Δ , 1,3- β -Glucanase activity without addition of xylose; $\blacktriangle$, 1,3- β -glucanase activity after addition of 0.133 M-xylose at 144 h; $\blacksquare$, 1,3- β -glucanase activity after addition of 0.066 M-xylose at 229 h; $\bullet$, 1,3- β -glucanase activity after addition of 0.133 M-xylose at 229 h; $\circ$, residual glucose concentration in the culture supernatant. Values are means of at least two independent experiments.

Fig. 5

Fig. 6

Fig. 5. Repression of extracellular 1,6- β -glucanase activity by xylose. Cultivation conditions were as in Fig. 4. After 142 h 0.133 M and after 165 h 0.066 M and 0.133 M-xylose was added (arrowed). $\square$, 1,6- β -Glucanase activity without addition of xylose; $\blacksquare$, 1,6- β -glucanase activity after addition of 0.133 M-xylose at 142 h; $\bullet$, 1,6- β -glucanase activity after addition of 0.066 M-xylose at 165 h; $\blacktriangle$, 1,6- β -glucanase activity after addition of 0.133 M-xylose at 165 h; $\circ$, residual glucose concentration in the culture supernatant. Values are means of at least two independent experiments.

Fig. 6. Repression of extracellular β -glucosidase activity by xylose. Cultivation conditions were as in Fig. 4. After 142 h 0.133 M and after 165 h 0.066 M and 0.133 M-xylose was added (arrowed). ∇ , β -Glucosidase activity without addition of xylose; $\blacktriangledown$, β -glucosidase activity after addition of 0.133 M-xylose at 142 h; $\blacksquare$, β -glucosidase activity after addition of 0.066 M-xylose at 165 h; $\bullet$, β -glucosidase activity after addition of 0.133 M-xylose at 165 h; $\circ$, residual glucose concentration in the culture supernatant. Values are means of at least two independent experiments.

Inhibition of extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities

Data on the inhibition of the three extracellular enzyme activities by glucose and glucono- δ -lactone and the corresponding K_i values are summarized in Table 2. Inhibition of β -glucosidase activity clearly differed from that of the two β -glucanase activities. 1,3- β - and 1,6- β -glucanase activities were uncompetitively inhibited by glucose and glucono- δ -lactone,

Table 2. *Inhibition of extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities by glucose and glucono- δ -lactone*

Values are means of at least three independent experiments.

Enzyme activity*	Inhibitor	Inhibition	K_i (mM)
1,3- β -Glucanase	Glucose	Uncompetitive	8.67
	Glucono- δ -lactone	Uncompetitive	3.17
1,6- β -Glucanase	Glucose	Uncompetitive	6.81
	Glucono- δ -lactone	Uncompetitive	12.79
β -Glucosidase	Glucose	Noncompetitive	1.89
	Glucono- δ -lactone	Noncompetitive	0.035

* The crude enzyme solutions used had specific activities as follows: 1,3- β -glucanase, 4.4 to 6.0 U (mg protein)⁻¹; 1,6- β -glucanase, 5.5 U (mg protein)⁻¹; β -glucosidase, 1.9 to 3.4 U (mg protein)⁻¹.

whereas β -glucosidase activity was noncompetitively inhibited. Moreover, β -glucosidase activity was more strongly inhibited, especially by glucono- δ -lactone, than the two β -glucanase activities. The effect of 1 mM-Hg²⁺, 1 mM-Pb²⁺, 1 mM-*p*-hydroxymercuribenzoate, 1 mM-ethyl mercurithiosalicylate and 1 and 10 mM-EDTA on β -glucanase and β -glucosidase activities was also studied. Extracellular 1,3- β - and 1,6- β -glucanase as well as β -glucosidase activity were inhibited only by Hg²⁺ (40 to 65% inhibition).

Effect of pH value and temperature on extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities

1,3- β - and 1,6- β -glucanase activities had the same pH optimum (4.0), while β -glucosidase activity was greatest at pH 5.0 (Fig. 7). 1,3- β -Glucanase and β -glucosidase activities had high temperature optima – 70 and 75 °C, respectively – whereas the optimum temperature for 1,6- β -glucanase activity was only 50 °C (Fig. 8). The rate of heat inactivation of β -glucanase and β -glucosidase activities was determined by incubating crude enzyme solutions at various temperatures. 1,3- β -Glucanase lost 33% of its activity after incubation for 30 min at 60 °C and only 3% of activity was left when the enzyme solution was heated for 30 min at 70 °C. After incubation for 30 min at 60 °C and 70 °C, 1,6- β -glucanase activity was reduced by 47 and 91%, respectively. β -Glucosidase activity was reduced by 38% after heating for 30 min at 60 °C and was completely inactivated after 25 min at 70 °C.

Interaction of Concanavalin A with extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities

Information on the glycosylation of the extracellular enzymes was obtained by examining their capacity to bind to Con A–Sephadex. Samples from supernatants of shake-flask cultures (250 ml culture in a 1 litre shake-flask; 100 r.p.m.) grown on 1% (w/v) glucose for 144 to 264 h were used as crude enzyme solutions. Differences in the extent of binding of β -glucosidase and the two β -glucanase activities were found throughout the cultivation period. Thus Con A bound 94% of extracellular β -glucosidase activity, but only about 33% of extracellular 1,3- β -glucanase activity and about 35% of extracellular 1,6- β -glucanase activity.

Degradation of extracellular 1,3- β -/1,6- β -glucan by culture supernatants containing 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities

Extracellular 1,3- β -/1,6- β -glucan, produced during cultivation, was incompletely degraded when the culture was depleted of carbon source (Fig. 1). After 210 h cultivation with an initial glucose concentration of 1% (w/v) 86% of the extracellular β -glucan had been hydrolysed. This percentage did not increase on further cultivation. In order to determine whether extracellular enzymes were responsible for hydrolysis, culture supernatants were used. Polysaccharide degradation was followed in two reaction mixtures both containing 5.4 mg purified extracellular β -glucan ml⁻¹ and 2.95 U 1,3- β -glucanase activity ml⁻¹. The mixtures differed in 1,6- β -glucanase and β -glucosidase activity. Reaction mixture I contained 0.4 U 1,6- β -glucanase

Fig. 7

Fig. 7. Influence of pH on extracellular 1,3- β -glucanase (Δ), 1,6- β -glucanase ($\square$) and β -glucosidase activities (∇).

Fig. 8

Fig. 8. Influence of temperature on extracellular 1,3- β -glucanase (Δ), 1,6- β -glucanase ($\square$) and β -glucosidase activities (∇). Values are means of at least two independent experiments.

ml⁻¹ and 0.16 U β -glucosidase activity ml⁻¹; mixture II contained 0.23 U 1,6- β -glucanase ml⁻¹ and 0.19 U β -glucosidase activity ml⁻¹. The most striking difference between both mixtures was their activity against periodate-oxidized laminarin. Reaction mixture I contained 2.0 U ml⁻¹ of activity against periodate-oxidized laminarin but mixture II only 0.45 U ml⁻¹. The two mixtures and the controls, which consisted of 1,3- β -/1,6- β -glucan solution and heat-inactivated culture supernatants, were incubated at 27 °C. In reaction mixture I, 64, 77 and 81% of β -glucan was degraded after 1, 3 and 24 h incubation, respectively. In mixture II, 54, 56 and 64% of the polysaccharide was hydrolysed after the same time. With periodate-oxidized laminarin as substrate, endo-1,3- β -glucanase activity can be determined only in the absence of exo-1,3- β -glucanase activity. Nevertheless, the higher activity towards periodate-oxidized laminarin in reaction mixture I may possibly imply a higher endo-1,3- β -glucanase activity, which in turn may be responsible for the increased degradation of extracellular β -glucan. Since the extent of polysaccharide degradation in cultures and supernatants was very similar (86 and 81%, respectively), it can be concluded that during cultivation the excreted 1,3- β -/1,6- β -glucan was almost exclusively hydrolysed by extracellular enzymes.

DISCUSSION

Under conditions of carbon-source limitation significant amounts of extracellular and low levels of intracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities were produced by *S. glucanicum*. The different time-course and extent of intra- and extracellular enzyme production indicate that the three enzyme activities were exported. A low intracellular

level of 1,3- β -glucanase activity compared with extracellular activity was also measured during cultivation of the Basidiomycete species QM 806. Moreover, immunodiffusion of cell-free extracts against antibodies to the pure extracellular 1,3- β -glucanase gave a scarcely detectable precipitin band, thus excluding the intracellular storage of a 1,3- β -glucanase with the enzymic and immunological properties of the extracellular enzyme (Friebe & Holldorf, 1975). The intracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities of *S. gluconicum* may have originated from vesicles which were ruptured during mechanical disintegration of mycelium. Vesicles containing 1,3- β -glucanase have been found in the fungi *Phytophthora palmivora* (Meyer *et al.*, 1976) and *Saprolegnia monoica* (Fèvre, 1979). Although the cells of *S. gluconicum* were thoroughly washed prior to cell disruption, the possibility cannot be excluded that some of the mycelium-bound enzyme activity was released during preparation of the cell-free extract. However, this contribution would have been very small, since mycelium-bound 1,3- β - and 1,6- β -glucanase activities were low compared with the intracellular enzyme activities.

Although there is no direct evidence of the involvement of lytic enzymes in hyphal growth, it is tacitly assumed that the plasticity of the growing hyphal apex is the result of a delicate balance between wall synthesis and the action of wall lytic enzymes (Bartnicki-García & Lippmann, 1972; Bartnicki-García, 1973). However, the presumed function of known glucanases during hyphal tip growth has been questioned (Del Rey *et al.*, 1979; Santos *et al.*, 1979; Molina *et al.*, 1987; Cenamor *et al.*, 1987). Similarly, 1,3- β - and 1,6- β -glucan-degrading enzymes do not play any role in the concept of Sietsma *et al.* (1985) of wall glucan synthesis during apical extension growth of *S. commune*. When *S. gluconicum* was actively growing trace amounts of 1,3- β - and 1,6- β -glucanase activities were found to be bound to mycelium and low levels of β -glucanase activities were found associated with cells and/or water-soluble 1,3- β -/1,6- β -glucan adhering to the fungal walls. Very small intracellular 1,3- β -glucanase activities were measured during hyphal growth of *S. gluconicum*. The possibility cannot be excluded that these intracellular and cell-bound enzyme activities, despite the low levels detected, play a role, perhaps not in apical extension, but in organizing centres for subsequent branch formation in already rigidified cell walls. Alternatively, the 1,3- β - and 1,6- β -glucanase activities detected during growth of *S. gluconicum* may represent the basal level of the constitutive β -glucanases, whose synthesis is repressed by glucose and other readily utilizable carbon sources. Almost negligible amounts of intra- and extracellular 1,3- β - and 1,6- β -glucanase activities were also synthesized during growth of *Neurospora crassa* in glucose-supplemented medium (Del Rey *et al.*, 1979).

The synthesis of extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities in *S. gluconicum* is regulated by catabolite repression. This form of control of β -glucanase formation has been observed in other fungi including *N. crassa* (Del Rey *et al.*, 1979), *Penicillium italicum* (Santos *et al.*, 1977), the Basidiomycete QM 806 (Friebe & Holldorf, 1975), *S. rolsfii* (Stephan, 1987) and *S. commune* (Wessels, 1966; Münzer, 1989). It is tempting to assume that cyclic adenosine 5'-monophosphate is involved in catabolite repression in fungi. However, unlike the situation in *Escherichia coli*, catabolite repression in the yeast *Saccharomyces cerevisiae* is not linked to cAMP (Johnston, 1987).

From experiments with cycloheximide we concluded that protein synthesis is necessary for the appearance of intracellular, cell-bound and extracellular 1,3- β -glucanase, 1,6- β -glucanase and β -glucosidase activities in derepressed cells of *S. gluconicum*. *De novo* synthesis was also suggested for the 1,3- β -glucanases of *P. italicum* (Santos *et al.*, 1978), three enzymes of which were detected in the cytoplasm as well as in culture fluid, with only two being bound to the cell wall (Santos *et al.*, 1979). In the case of *S. gluconicum* it is not known whether the extracellular β -glucan-degrading enzymes correspond in number and character to the cell-bound and/or intracellular β -glucanases.

Derepression of β -glucanase formation in *S. gluconicum* during incubation of shake-flask cultures deprived of glucose involved extensive autolysis of mycelium and degradation of almost 90% of the extracellular 1,3- β -/1,6- β -glucan. The latter is hydrolysed by extracellular β -glucanases and the fungal walls are possibly degraded by mycelium-bound β -glucanases. β -Glucanase formation by resting cells of *Claviceps fusiformis* (Dickerson *et al.*, 1970), *P. italicum* (Santos *et al.*, 1979), *S. commune* (Wessels & Sietsma, 1979; Münzer, 1989) and *S. rolsfii*

(Stephan, 1987) could also be linked to mobilization of cell walls and/or extracellular β -glucan. Degradation of fungal cell wall constituents and extracellular glucan is often related to morphogenetic events such as formation of cleistothecia (Zonneveld, 1972), pilei (Wessels & Sietsma, 1979; Kamada *et al.*, 1982, 1985) and sclerotia (Christias & Lockwood, 1973; Hadar *et al.*, 1983). A relationship between mobilization of cell wall or extracellular β -glucan and formation of sclerotia or conidiation in *S. glaucanicum* has not been observed to date.

The inhibition of extracellular β -glucosidase activity from *S. glaucanicum* by glucose and glucono- δ -lactone differed from those of the extracellular 1,3- β - and 1,6- β -glucanase activities not only in its mode but also in its extent. The results obtained agree with those of Reese *et al.* (1971) in that extracellular β -glucosidase activity of *S. glaucanicum* was more strongly inhibited by glucono- δ -lactone than were the extracellular 1,3- β - and 1,6- β -glucanase activities. The lack of inhibition of β -glucanase and β -glucosidase activity by *p*-hydroxymercuribenzoate and ethyl mercurithiosalicylate and the moderate inhibition of these enzyme activities by Hg^{2+} suggests either the absence or poor accessibility of -SH groups essential for the three enzyme activities.

Laminarin and pustulan are commonly used as substrates for the assay of 1,3- β - and 1,6- β -glucanase activities (Reese & Mandels, 1966), but activity against laminarin and pustulan does not necessarily reflect the ability of enzymes to attack high molecular mass 1,3- β -/1,6- β -glucans. Purification of extracellular β -glucanases from *S. glaucanicum* (the results of which will be reported elsewhere) has shown the presence of one endo-1,3- β - and several exo-1,3- β -glucanases. The endoglucanase hydrolysed not only laminarin but also water-soluble 1,3- β -/1,6- β -glucan of high molecular mass. The exoglucanases were only tested for the ability to split laminarin and it can only be assumed that some of them attack high molecular mass 1,3- β -/1,6- β -glucans.

Almost all of the total extracellular β -glucosidase activity but only one-third of the 1,3- β - and 1,6- β -glucanase activities could be bound to Con A-Sepharose. Based on this binding capacity and the mode of inhibition of extracellular β -glucosidase activity, which differ from those of 1,3- β - and 1,6- β -glucanase activities, β -glucosidase activity can be attributed to a separate exo- β -glucanase or β -glucosidase enzyme. Extracellular hydrolases of fungi are normally glycosylated (Yoshizumi & Ashikari, 1987). The finding that only about one-third of the extracellular 1,3- β - and 1,6- β -glucanase activities from *S. glaucanicum* interacted with Concanavalin A does not prove that some of the endo- and exoglucanases are not glycosylated. Two purified exo-1,3- β -glucanases from *Pichia polymorpha* failed to bind to Concanavalin A, despite their D-glucose and D-mannose contents (Villa *et al.*, 1978). Enzymes may fail to bind to Con A-Sepharose because the number of receptor sites in the carbohydrate moiety or its spatial configuration are inadequate for effective binding to the lectin (Phaff, 1979). On the other hand, it cannot be excluded that some β -glucanases from *S. glaucanicum* are not glycosylated, as was shown for an endo-1,3- β -glucanase from *Schizosaccharomyces versatilis* (Fleet & Phaff, 1974).

The skilful technical assistance of Mrs B. Müller was very much appreciated.

This paper is dedicated to Professor Saburo Fukui, Kyoto, Japan, on the occasion of his 70th birthday.

REFERENCES

- BARTNICKI-GARCÍA, S. (1973). Fundamental aspects of hyphal morphogenesis. *Symposia of the Society for General Microbiology* **23**, 245-267.
- BARTNICKI-GARCÍA, S. & LIPPMAN, E. (1972). The bursting tendency of hyphal tips of fungi: presumptive evidence for a delicate balance between cell wall synthesis and wall lysis in apical growth. *Journal of General Microbiology* **73**, 487-500.
- BLUHM, T. L., DESLANDES, Y., MARCHESSAULT, R. H., PÉREZ, S. & RINAUDO, M. (1982). Solid-state and solution conformation of scleroglucan. *Carbohydrate Research* **100**, 117-130.
- BRUNER, R. L. (1964). Determination of reducing value. 3,5-Dinitrosalicylic acid method. *Methods in Carbohydrate Chemistry* **4**, 67-71.
- CATLEY, B. J. (1983). Regulation of yeast and fungal polysaccharides excluding chitin and cellulose. *Progress in Industrial Microbiology* **18**, 129-200.
- CENAMOR, R., MOLINA, M., GALDONA, J., SÁNCHEZ, M. & NOMBELA, C. (1987). Production and secretion of *Saccharomyces cerevisiae* β -glucanases: differences between protoplast and periplasmic enzymes. *Journal of General Microbiology* **133**, 619-628.
- CHESTERS, C. G. C. & BULL, A. T. (1963). The enzymic degradation of laminarin. 1. The distribution of laminarinase among microorganisms. *Biochemical Journal* **86**, 28-31.
- CHRISTIAS, C. & LOCKWOOD, J. L. (1973). Conservation of mycelial constituents in four sclerotium-forming fungi in nutrient-deprived conditions. *Phytopathology* **63**, 602-605.
- DAVIS, E. N., RHODES, R. A. & RUSSELL SHULKE, H.

- (1965). Fermentative production of exocellular glucans by fleshy fungi. *Applied Microbiology* **13**, 267–271.
- DEL REY, F., GARCÍA-ACHA, I. & NOMBELA, C. A. (1979). The regulation of β -glucanase synthesis in fungi and yeast. *Journal of General Microbiology* **110**, 83–89.
- DICKERSON, A. G., MANTLE, P. G. & SZCZYRBAK, C. A. (1970). Autolysis of extracellular glucans produced *in vitro* by a strain of *Claviceps fusiformis*. *Journal of General Microbiology* **60**, 403–415.
- FÈVRE, M. (1979). Glucanases, glucan synthases and wall growth in *Saprolegnia monoica*. In *Fungal Walls and Hyphal Growth*, pp. 225–263. Edited by J. H. Burnett & A. P. J. Trinci. Cambridge: Cambridge University Press.
- FLEET, G. H. & PHAFF, H. J. (1974). Glucanases in *Schizosaccharomyces*. Isolation and properties of the cell wall-associated $\beta(1\rightarrow3)$ -glucanases. *Journal of Biological Chemistry* **249**, 1717–1728.
- FRIEBE, B. & HOLLDORF, A. W. (1975). Control of extracellular β -1,3-glucanase activity in a basidiomycete species. *Journal of Bacteriology* **122**, 818–825.
- GOLDSTEIN, I. J., HAY, G. W., LEWIS, B. A. & SMITH, F. (1965). Controlled degradation of polysaccharides by periodate oxidation, reduction and hydrolysis. *Methods in Carbohydrate Chemistry* **5**, 361–369.
- HADAR, Y., PINES, M., CHET, I. & HENIS, Y. (1983). The regulation of sclerotium initiation in *Sclerotium rolfsii* by glucose and cyclic AMP. *Canadian Journal of Microbiology* **29**, 21–26.
- HALLECK, F. E. (1967). Polysaccharides and methods for production thereof. US Patent 3301848.
- JOHNSON, J., JR, KIRKWOOD, S., MISAKI, A., NELSON, T. E., SCALETTI, J. V. & SMITH, F. (1963). Structure of a new glucan. *Chemistry and Industry* 820–822.
- JOHNSTON, M. (1987). A model fungal gene regulatory mechanism: the *gal* genes of *Saccharomyces cerevisiae*. *Microbiological Reviews* **51**, 458–476.
- KAMADA, T., HAMADA, Y. & TAKEMARU, T. (1982). Autolysis *in vitro* of the stipe cell wall in *Coprinus macrorrhizus*. *Journal of General Microbiology* **128**, 1041–1046.
- KAMADA, T., FUJII, T., NAKAGAWA, T. & TAKEMARU, T. (1985). Changes in $(1\rightarrow3)$ - β -glucanase activities during stipe elongation in *Coprinus cinereus*. *Current Microbiology* **12**, 257–260.
- KIKUMOTO, S., MIYAJIMA, T., YOSHIKUNI, S., FUJIMOTO, S. & KIMURA, K. (1970). Polysaccharide produced by *Schizophyllum commune*. I. Formation and some properties of an extracellular polysaccharide. *Nippon Nogei Kagaku Kaishi* **44**, 337–342.
- KIKUMOTO, S., MIYAJIMA, T., KIMURA, K., OKUBO, S. & KOMATSU, N. (1971). Polysaccharide produced by *Schizophyllum commune*. II. Chemical structure of an extracellular polysaccharide. *Nippon Nogei Kagaku Kaishi* **45**, 162–168.
- KOTTUTZ, E. (1989). *Untersuchungen zur in vivo- und in vitro- β -Glucan-Synthese bei Sclerotium glaucum CBS 52071*. PhD thesis, Technische Universität Braunschweig, FRG.
- MEYER, R., PARISH, R. W. & HOHL, H. R. (1976). Hyphal tip growth in *Phytophthora*. Gradient distribution and ultra-histochemistry of enzymes. *Archives of Microbiology* **110**, 215–224.
- MILLER, G. L., BLUM, R., GLENNON, W. E. & BURTON, A. L. (1960). Measurement of carboxymethyl-cellulase activity. *Analytical Biochemistry* **2**, 127–132.
- MOLINA, M., CENAMOR, R. & NOMBELA, C. (1987). Exo-1,3- β -glucanase activity in *Candida albicans*: effect of the yeast-to-mycelium transition. *Journal of General Microbiology* **133**, 609–617.
- MÜNZER, S. (1989). *Produktion und Charakterisierung eines von Schizophyllum commune ATCC 38548 gebildeten extrazellulären β -1,3-Glucans*. PhD thesis, Technische Universität Braunschweig, FRG.
- NACHTWEY, S. (1989). *Bildung und partielle Charakterisierung eines extrazellulären Polysaccharids von Monilinia fructicola*. PhD thesis, Technische Universität Braunschweig, FRG.
- NIEDERPRUEM, D. J., MARSHALL, C. & SPETH, J. L. (1977). Control of extracellular slime accumulation in monokaryons and resultant dikaryons of *Schizophyllum commune*. *Sabouraudia* **15**, 283–295.
- PHAFF, H. J. (1979). A retrospective and current view on endogenous β -glucanases in yeast. In *Advances in Protoplast Research (Proceedings of the 5th International Protoplast Symposium)*, pp. 171–182. Budapest: Hungarian Academy of Sciences.
- RAPP, P., BECK, C. H. & WAGNER, F. (1979). Formation of exopolysaccharides by *Rhodococcus erythropolis* and partial characterization of a heteropolysaccharide of high molecular weight. *European Journal of Applied Microbiology and Biotechnology* **7**, 67–78.
- REESE, E. T. (1977). Degradation of polymeric carbohydrates by microbial enzymes. *Recent Advances in Phytochemistry* **11**, 311–367.
- REESE, E. T. & MANDELS, M. (1959). β -1,3-Glucanases in fungi. *Canadian Journal of Microbiology* **5**, 173–185.
- REESE, E. T. & MANDELS, M. (1966). β -Glucanases other than cellulase. *Methods in Enzymology* **8**, 607–615.
- REESE, E. T., PARRISH, F. W. & ETTLINGER, M. (1971). Nojirimycin and D-glucono-1,5-lactone as inhibitors of carbohydrases. *Carbohydrate Research* **18**, 381–388.
- REESE, E. T., PARRISH, F. W. & MANDELS, M. (1962). β -1,6-Glucanases in fungi. *Canadian Journal of Microbiology* **8**, 327–334.
- RINAUDO, M. & VINCENDON, M. (1982). ^{13}C NMR structural investigation of scleroglucan. *Carbohydrate Polymers* **2**, 135–144.
- SANTOS, T., VILLANUEVA, J. R. & NOMBELA, C. (1977). Production and catabolite repression of *Penicillium italicum* β -glucanases. *Journal of Bacteriology* **129**, 52–58.
- SANTOS, T., VILLANUEVA, J. R. & NOMBELA, C. (1978). Regulation of β -1,3-glucanase synthesis in *Penicillium italicum*. *Journal of Bacteriology* **133**, 542–548.
- SANTOS, T., SÁNCHEZ, M., VILLANUEVA, J. R. & NOMBELA, C. (1979). Derepression of β -1,3-glucanases in *Penicillium italicum*: localization of the various enzymes and correlation with cell wall glucan mobilization and autolysis. *Journal of Bacteriology* **137**, 6–12.
- SIETSMAN, J. H., SONNENBERG, A. M. S. & WESSELS, J. G. H. (1985). Localization by autoradiography of synthesis of $(1\rightarrow3)$ - β and $(1\rightarrow6)$ - β linkages in a wall glucan during hyphal growth of *Schizophyllum commune*. *Journal of General Microbiology* **131**, 1331–1337.

- SINGH, P. P., WHISTLER, R. L., TOKUZEN, R. & NAKAHARA, W. (1974). Scleroglucan, an antitumor polysaccharide from *Sclerotium glaucum*. *Carbohydrate Research* **37**, 245–247.
- STEPHAN, D. (1987). *Bildung von β -1,3-Glucanen aus Mono-, Di- und Polysacchariden mit Sclerotium rolfsii und deren physikochemische Charakterisierung*. PhD thesis, Technische Universität Braunschweig, FRG.
- STOPPOK, W., RAPP, P. & WAGNER, F. (1982). Formation, localization and regulation of endo-1,4- β -glucanases and β -glucosidases from *Cellulomonas uda*. *Applied and Environmental Microbiology* **44**, 44–53.
- VILLA, T. G., NOTARIO, V. & VILLANUEVA, J. R. (1978). Direct chemical proof of different glycosylation patterns for yeast exo- and endo-1,3- β -glucanases. *Journal of General Microbiology* **109**, 371–374.
- WESSELS, J. G. H. (1966). Control of cell-wall glucan degradation during development in *Schizophyllum commune*. *Antonie van Leeuwenhoek* **32**, 341–355.
- WESSELS, J. G. H. & SIETSMAN, J. H. (1979). Wall structure and growth in *Schizophyllum commune*. In *Fungal Walls and Hyphal Growth*, pp. 27–48. Edited by J. H. Burnett & A. P. J. Trinci. Cambridge: Cambridge University Press.
- YANAKI, T., KOJIMA, T. & NORISUYE, T. (1981). Triple helix of scleroglucan in dilute aqueous sodium hydroxide. *Polymer Journal* **13**, 1135–1143.
- YOSHIZUMI, H. & ASHIKARI, T. (1987). Expression, glycosylation and secretion of fungal hydrolases in yeast. *Trends in Biotechnology* **5**, 277–281.
- ZONNEVELD, B. J. M. (1972). Morphogenesis in *Aspergillus nidulans*. The significance of α -1,3-glucan of the cell wall and α -1,3-glucanase for cleistothecium development. *Biochimica et biophysica acta* **273**, 174–187.