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Formation and Location of 1,4-β-Glucanases and 1,4-β-Glucosidases from *Penicillium janthinellum*

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Formation and location of 1,4-β-glucanases and 1,4-β-glucosidases were studied in cultures of *Penicillium janthinellum* grown on Avicel, sodium carboxymethyl cellulose, cellobiose, glucose, mannose, and maltose. Endo-1,4-β-glucanases were found to be cell free, and their formation was induced by cellobiose. 1,4-β-Glucosidases, on the other hand, were formed constitutively and were primarily cell free, but with a small amount strongly associated with the cell wall. Low 1,4-β-glucosidase activities of periplasmic or intracellular origin were also found. A rotational viscosimetric method was developed to measure the total endo-1,4-β-glucanase activity of the culture (broth and solids). By this method, it was possible to determine the endo-1,4-β-glucanase activity not only in the supernatant of the culture but also on the surface of the mycelium or absorbed on residual Avicel. During a 70-liter batch cultivation of *P. janthinellum*, the adsorption of endo-1,4-β-glucanases by residual and newly added 10% Avicel was measured. The adsorption of soluble protein and endo-1,4-β-glucanases by Avicel was found to be largely independent of the pH value but dependent on temperature.

Cellulose, a highly polymeric, often crystalline substrate, cannot penetrate the cell wall and therefore must be degraded outside the cell. This microbial degradation takes place by a mixture of at least three kinds of enzymes. Endo-1,4-β-glucanase (EC 3.2.1.4) attacks at random the 1,4-β-linkages along a cellulose chain, whereas the exo-1,4-β-glucanase (EC 3.2.1.91) splits off cellobiose from the nonreducing chain end. 1,4-β-Glucosidase (EC 3.2.1.21) finally hydrolyzes cellobiose to glucose.

Cellulolytic *Penicillium* species include *P. citrinum* (23), *P. funiculosum* (27), *P. iriense* (8), *P. notatum* (2, 25, 26), *P. variable* (4), and an unidentified *Penicillium* strain, isolated by Bastawde et al. (5). The location of cellulase enzymes is important in that since cellulose is insoluble, free enzyme is essential to gain efficient cellulose degradation. Location of the 1,4-β-glucosidases is similarly important, but these enzymes can act either in the cell-bound state or in solution. Another possible prerequisite for an effective degradation of cellulose, at least at the beginning of hydrolysis, is the adsorption of the glucanases on the surface of the cellulose fiber. This paper is concerned, therefore, with studies on the formation and location of 1,4-β-glucanases and 1,4-β-glucosidases in cultures of *P. janthinellum* grown on celluloses and several noncellulosic carbon sources. Furthermore, the induction of endo-1,4-β-glucanases and their adsorption on Avicel, a microcrystalline cellulose, is described.

MATERIALS AND METHODS

Organism. The fungus was isolated from forest soil and identified by Centraalbureau voor Schimmelcultures in Baarn, The Netherlands, as *P. janthinellum*.

Chemicals. All chemicals were analytical grade. Carboxymethyl cellulose (CM-cellulose) was type 132 from Schleicher & Schüll, Dassel, West Germany, with an exchange capacity of 1.05 mV1/g; Avicel no. 2331 was from E. Merck AG, Darmstadt, West Germany; sodium CM-cellulose (Na-CM-cellulose) was type 16110 from Serva, Heidelberg, West Germany.

Culture conditions. Stock cultures were maintained at 4°C and subcultured every 8 weeks by incubation at 27°C for 14 days. The medium for the stock cultures comprised (in grams per liter of deionized water): malt extract, 20; glucose, 20; peptone (Difco Laboratories, Detroit, Mich.), 1; Avicel, 20; and agar (Difco), 20. The pH value before autoclaving was adjusted to 5.5.

Submerged cultures were grown in the following basal medium (in grams per liter of deionized water): $K_2HPO_4 \cdot 3H_2O$, 1.0; KH_2PO_4, 0.5; $Na_2HPO_4 \cdot 2H_2O$, 0.5; $MgSO_4$, 0.3; and $(NH_4)_2SO_4$, 2.8. $CaCl_2 \cdot 2H_2O$, 0.3, was added aseptically, as was 1 ml of a trace metal stock solution. The trace metal stock solution consisted of (m grams per liter of distilled water): $MnSO_4 \cdot H_2O$, 1.56; $FeSO_4 \cdot 7H_2O$, 5.0; $ZnCl_2$, 1.67; and $CoCl_2 \cdot 6H_2O$, 2.0.

A spore inoculum was prepared by adding about 2 ml of sterile 0.9% (wt/vol) NaCl solution to a 14-day-old slant and using it to inoculate basal medium (100/

500-ml shake flask; baffled Erlenmeyer flask) supplemented with 1.5% (wt/vol) of the appropriate carbon source. Cultures were grown at 30°C on a rotary shaker (100 rpm) for 2 days. The pH value was adjusted daily to 5.0 by adding sterile 1 N NaOH or H_3PO_4.

Batch cultivation in 1-liter shake flasks was performed under the conditions used for preparation of the first seed. Five milliliters of this first seed was inoculated into 250 ml of basal medium with 1.5% (wt/vol) of a corresponding carbon source and cultured for 2 or 7 days.

For 10-liter batch cultivations, 250 ml of the second seed, grown for 2 days on a given carbon source as described above, was inoculated into 9.75 liters of basal medium supplemented with a corresponding carbon source. They were carried out in a sparged and baffled 14-liter bioreactor (type b 10; Giovanola Frères S.A., Monthey, Switzerland) equipped with a turbine system with two sets of six flat-bladed impellers. The cultures were aerated at 0.5 vol/vol per min and stirred at 300 rpm. The pH value was automatically controlled at 5.0 by addition of 3.8 M NH_4OH.

For 70-liter batch cultivations, 3 liters of a second seed, cultivated as described above in shake flasks on basal medium with 1.5% (wt/vol) Avicel for 2 days, was inoculated into 67 liters of basal medium supplemented with 3% (wt/vol) Avicel. The cultivations were performed in an 80-liter bioreactor (type b 50, Giovanola Frères S.A.) equipped with a draught tube and driven by a specially constructed propeller (Intensor system, Giovanola Frères S.A.) at 1,500 rpm. The cultures were aerated at 0.18 vol/vol per min, and the pH value was automatically adjusted by titration with 14.7 M NH_4OH.

Determination of O_2 and CO_2 in the gas phase. O_2 and CO_2 in the exit stream of the bioreactor were monitored by Oxygor, a paramagnetic oxygen analyzer and Unor, a nondispersive infrared photometer (Maihak, Hamburg, West Germany). The range of O_2 concentrations for analysis was between 15 and 21%, and the measuring range of CO_2 concentrations was set from 0 to 5%.

Determination of cellulose. The cellulose content of cultures was determined by a shortened procedure of the method of Updegraff (31). Ten milliliters of the total culture was centrifuged (3,000 × *g* for 20 min), and the supernatant was carefully removed with a Pasteur pipette. The pellet was suspended in acetic acid-nitric acid reagent (3 ml; 150 ml of 80% acetic acid/15 ml of concentrated nitric acid) and boiled for 30 min in a water bath. After cooling and centrifuging (3,000 × *g* for 20 min), the pellet was washed with distilled water (10 ml), and the residual cellulose was dried at 40°C under reduced pressure to constant weight.

Determination of growth. The fungal growth on cellulose was followed by determining the nitrogen content of the mycelium by Kjeldahl analysis. The mycelial weight was calculated by assuming an average protein content of the mycelium of 29%; this conversion factor was found after growth on soluble carbon sources. Dry weight of the solids was also used to determine mycelial weight. By this method, the mycelial weight was calculated from the difference between the dry weight of the solids, comprising mycelium and residual cellulose, and the amount of residual cellulose, determined as described above. The dry weight of the solids was determined by centrifuging the culture (20 ml; 9,600 × *g* for 20 min), washing the pellet three times with water (10 ml), and drying at 40°C under reduced pressure to constant weight. Both methods probably give, at the beginning of growth, mycelial weights which are somewhat too high because of adsorption of glucanases and other proteins on the residual cellulose.

For soluble carbon sources, cell growth was determined by centrifuging the culture (20 ml; 9,600 × *g* for 20 min). The pellet was washed three times with water (10 ml) and dried at 40°C under reduced pressure to constant weight.

Determination of soluble protein. Soluble protein was determined according to Lowry et al. (16). A standard curve was prepared from determinations with bovine serum albumin.

Enzyme assays. 1,4-β-Glucanase activity toward CM-cellulose was determined by the colorimetric method of Miller et al. (20). The reaction mixture contained 1 ml of 1% (wt/vol) CM-cellulose in 0.01 M sodium citrate-phosphate buffer, pH 4.0, and enzyme solution (1 ml). After the mixture was incubated at 59°C for 15 min and the reaction was stopped by cooling in an ice bath, 3,5-dinitrosalicylic acid reagent (3 ml) was added and well mixed with a Vortex mixer. The reaction mixture was then heated for 12 min in a boiling water bath, and, after cooling in an ice bath, the absorbance was measured at 640 nm. A blank was always used with 0.01 M sodium citrate-phosphate buffer, pH 4.0 (1 ml), instead of enzyme solution (1 ml) to correct for reducing sugars in the substrate. One unit of CM-cellulose hydrolyzing activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min under the given conditions.

The 1,4-β-glucanase activity toward Avicel was also determined by measuring the amount of reducing sugar produced from Avicel. The reaction mixture contained Avicel (30 mg) in 0.01 M sodium acetate buffer, pH 4.0 (4 ml), and enzyme solution (1 ml). After incubating the mixture at 45°C for 60 min with shaking at 150 rpm, the reaction was stopped by cooling in an ice bath. The mixture was filtered, and 3,5-dinitrosalicylic acid (3 ml) was added to the filtrate (2 ml), thoroughly mixed with a Vortex mixer, and heated in a boiling water bath for 12 min. After cooling in an ice bath, the absorbance was measured at 640 nm. The blank contained, instead of the enzyme solution, 0.01 M sodium acetate buffer, pH 4.0 (1 ml), to correct for reducing sugars in the substrate. One unit of Avicel hydrolyzing activity was defined as the amount of enzyme which liberated 1 μmol of reducing sugar per min under the described conditions.

The rotational viscosimetric measurement of endo-1,4-β-glucanase activity was carried out as follows. The Na-CM-cellulose solution was prepared by dissolving Na-CM-cellulose (22 g) in 0.01 M sodium citrate-phosphate buffer, pH 4.0 (1 liter). After boiling for 2 to 3 min and filtering through a sieve plate to remove gel particles, a stable polymer solution was obtained with a final pH value of 4.2 and an average viscosity of about 150 mPa·s at 40°C. The stock solution was prepared fresh daily and thermostated at

40°C. The assays were carried out at 40°C in a Rotovisco RV 3 rotational viscosimeter with an MK 50 measuring head and MVII/MVSt rotor/stator system (Haake, Karlsruhe, West Germany). The Na-CM-cellulose solution (47 ml) was placed in the stator, and the initial viscosity was measured. Then the endoglucanase-containing supernatant or total culture (0.1 to 2.0 ml) was added and quickly mixed by shaking the stator for 30 s. The stator was replaced in the viscosimeter and incubated for 4.5 min at a constant speed of 181 rpm. Because the incubation time t was small (14), relative units were calculated by the rate of increase of reciprocal specific viscosity:

$$\eta_{sp_1} \frac{d}{dt}\left(\frac{1}{\eta_{sp}}\right) = \frac{\eta_{sp_1} - \eta_{sp_2}}{\eta_{sp_1} \cdot \eta_{sp_2}} \cdot \frac{1}{t}$$

The volume of the sample ws chosen to be between 0.1 and 2.0 ml so that the viscosity difference $\eta_{sp_1} - \eta_{sp_2}$ did not exceed 40 mPa·s.

1,4-β-Glucosidase activity was determined with *p*-nitrophenyl-β-D-glucoside as a substrate (7). The reaction mixture contained 1 mM *p*-nitrophenyl-β-D-glucoside in 0.025 M sodium acetate buffer, pH 5.0 (1 ml), and enzyme solution (100 μl). After incubation at 40°C for 10 min, 1 M sodium carbonate solution (2 ml) was added. The mixture was then chilled to about 4°C to stop the reaction, and the absorbance was measured at 400 nm.

Determination of the 1,4-β-glucosidase activity of the total culture was carried out in the same way, but with an additional step. The chilled reaction mixture was centrifuged (3,000 × *g* for 15 min at 4°C) to remove mycelium and residual cellulose. The absorbance of the cooled supernatant was measured as before at 400 nm.

The cell-associated 1,4-β-glucosidase activity was calculated from the difference of the activities of the total culture and supernatant. One unit of 1,4-β-glucosidase activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucoside per min under the assay conditions. The 1,4-β-glucosidase activity was calculated as follows: micromoles of *p*-nitrophenol formed per minute = $(EV)/(tv\epsilon d)$, where E is the extinction at 400 nm; V is the total volume of the reaction mixture (in cubic centimeters); t is the reaction time (in minutes); v is the volume of the sample (in cubic centimeters); ϵ = 18.8 ($cm^2/\mu mol$); and d is the light path of the cuvette (in centimeters).

The assay for proteolytic activity was performed according to Tsai et al. (30), using casein-yellow (Calbiochem, San Diego, Calif.) as the substrate. Before assaying for protease activity, the samples were concentrated 10-fold by a Minicon-B 15 (Amicon Corp., Lexington, Mass.). One unit of the proteolytic activity was defined as the amount of enzyme which caused an increase in absorbance at 423 nm of 0.1/h under the given conditions.

Adsorption of endo-1,4-β-glucanases and protein by Avicel. The percentage of endo-1,4-β-glucanase activity adsorbed by residual Avicel was determined by rotational viscosimetry from the difference between the endoglucanase activity in the total culture and in the supernatant. To determine the percentage of endo-1,4-β-glucanase activity and protein concentration adsorbed by an excess of 10% (wt/vol) Avicel, 2 ml of enzyme solution which had a 1,4-β-glucanase activity toward CM-cellulose not exceeding 2 U/ml, and 1 g of Avicel were added to 0.01 M sodium citrate-phosphate buffer, pH 5.0 (8 ml). The flasks were incubated in a water bath with shaking for 30 min at 30°C. When the effect of pH on adsorption was studied, the same buffer was used, but with final pH values of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0. In assays of the influence of temperature on adsorption, 0.01 M sodium citrate-phosphate buffer, pH 5.0, was used. The samples were centrifuged, and the supernatants were assayed for endoglucanase activity and protein concentration. Adsorption was estimated by subtracting the residual enzyme activity and protein concentration in solution from the values for a similar sample, incubated at the same pH value and temperature without Avicel.

Desorption of endo-1,4-β-glucanases from residual Avicel. In the desorption experiments, a sample of the total culture (2 ml) containing, in addition to cell-free endoglucanases also those adsorbed by residual Avicel, was added to 2% (wt/vol) Na-CM-cellulose solution (8 ml), as used for the rotational viscosimetric measurement of endoglucanase activity, and stirred at 40°C for 5 min. After centrifugation, the endoglucanase activity in the supernatant was measured by rotational viscosimetry. From this amount, which comprised cell-free and desorbed endo-1,4-β-glucanase activity, the latter could be calculated by subtracting the endoglucanase activity measured in the supernatant of the culture.

Another experiment to assay the complete desorption of endoglucanases from residual Avicel was performed as follows. Endo-1,4-β-glucanases (0.04 relative U/ml) in sterile basal medium with 5% (wt/vol) Avicel (100/500-ml shake flasks) were incubated at 30°C on a rotary shaker (100 rpm) for 17 h. A control was run under the same conditions but without Avicel. At appropriate intervals, samples were taken and the endoglucanases were desorbed and measured as described above. The values obtained were compared with those of the control experiment carried out in the same manner.

Rotational viscosimetric method for determination of the induction of endo-1,4-β-glucanases. The method for studying the induction of endo-1,4-β-glucanases was similar to that of Eriksson and Hamp (10). Mycelial pellets used for the induction experiments were obtained by cultivating the fungus first in 100 ml of basal medium with 1% (wt/vol) glucose as the sole carbon source for 48 h. This culture (5 ml) was used to inoculate 250 ml of basal medium with 0.5% (wt/vol) glucose as the sole carbon source and grown for 29 h. The culture conditions were the same as described above. The pellets were harvested by sterile centrifugation and washed four times with sterile basal medium. Wet mycelium (4 g), corresponding to a dry weight of 0.15 g, was aseptically transferred into 1-liter shake flasks containing 250 ml of basal medium with 2% (wt/vol) Na-CM-cellulose, pH 5.0. Cellobiose or sophorose was added in known concentrations to the sterile Na-CM-cellulose medium without being sterilized to avoid degradation. These my-

celium-containing Na-CM-cellulose solutions, with and without added sugars, were incubated on a rotary shaker (100 rpm) at 30°C, and the viscosity was measured at corresponding intervals by the rotational viscosimetric method. To visualize the induction, the percentage decrease of the starting viscosity was plotted as function of the incubation time (see Fig. 5).

Ultrasonic disruption of the cells. The total culture (50 ml) was centrifuged (46,000 × *g* for 30 min at 4°C). The pellet was washed six times with 0.9% (wt/vol) NaCl solution (20 ml). To the pellet, which was free from extracellular 1,4-β-glucosidase activity, 0.025 M sodium acetate buffer, pH 5.0 (25 ml), was added, and the mycelium was disrupted ultrasonically for 15 min, under cooling in an ice bath, with a Sonifier (model J-17-A; Branson Sonic Power Co., Danbury, Conn.). A microtip was used, and the output control was set at 4. Samples were examined microscopically to confirm that the cell walls had been extensively ruptured.

RESULTS

Growth and formation of 1,4-β-glucanases and 1,4-β-glucosidases during cultivation on cellulose. *P. janthinellum* is a mesophilic plectomycete having an optimal temperature of 30°C and optimal pH of 5.0 for cellulase production. Figure 1 illustrates the growth of the fungus and the formation of cellulolytic enzymes during a 70-liter batch cultivation on 3% (wt/vol) Avicel as the sole carbon source. About 35 h after inoculation, remarkable excretion of 1,4-β-glucanases and 1,4-β-glucosidases into the culture medium started together with a rapid decrease of the cellulose content. The lag growth phase was reduced almost by half when the inoculum was cultivated in a bioreactor at a nearly constant pH instead of in shake flasks, where the pH was corrected only once a day. Within 140 h cellulose was almost completely degraded, with only 3 to 4% being left. The glucanase activities toward Avicel and CM-cellulose reached maximal amounts of 0.7 and 1.05 U/ml, respectively, after about 110 h of incubation, whereas the highest 1,4-β-glucosidase activity, 1.5 U/ml, was obtained later, after 130 h. Small amounts of cell-associated 1,4-β-glucosidase activity were measured over the total cultivation period. In the stationary growth phase, a mycelial dry weight of 8.0 g/liter was achieved. Also shown in Fig. 1 is the increase of soluble protein concentration and protease activity in the culture filtrate. The latter reached a maximum of 2.3 U/ml earlier than the cellulolytic enzymes, after about 70 h, and the highest content of soluble protein was 2.4 g/liter.

P. janthinellum growth and formation of cellulolytic enzymes were remarkably reduced when the fungus was cultured on 1.5% (wt/vol) Na-CM-cellulose. Fungal growth had already ceased about 40 h after inoculation, reaching only 1.4 g of mycelial dry weight per liter (Fig. 2). On the other hand, the stationary growth phase of a comparable batch culture on 1.5% (wt/vol) Avicel was reached in only twice the time but with 7 g of mycelial dry weight per liter (E. Grote, Ph.D. thesis, Universität Braunschweig, Braunschweig, West Germany, 1979). The 1,4-β-glucanase activity toward Avicel and CM-cellulose as well as the 1,4-β-glucosidase activity during growth on Na-CM-cellulose were only about 10% of those obtained during cultivation on 1.5% (wt/vol) Avicel (Fig. 2; Grote, Ph.D. thesis, 1979). In cultures grown on Na-CM-cellulose, small amounts of cell-associated β-glucosidase activity were also measured throughout the cultivation period.

Rotational viscosimetric determination of endo-1,4-β-glucanase activity in the total culture. The activity of endo-1,4-β-glucanases in the total culture of *P. janthinellum* grown on Avicel was determined by a rotational viscosimetric method, using an excess of Na-CM-cellulose as the substrate. The volume of the sample was small enough that the starting viscosity was not changed either by dilution or by addition of too many solids such as residual Avicel or mycelium. By adsorption of endoglucanases on 10% (wt/vol) Avicel and followed by desorption experiments of different lengths, we found that less than 5 min was sufficient for the complete desorption of endo-1,4-β-glucanases. This rapid and complete desorption of endo-1,4-β-glucanases by a large excess of Na-CM-cellulose was also tested for in partially degraded and structurally changed (i.e., residual) Avicel. For this purpose, endoglucanases in basal medium with and without 5% (wt/vol) Avicel were incubated for 17 h, and desorption experiments were carried out throughout the incubation period. We found that endoglucanases adsorbed on residual Avicel were also completely desorbed within 5 min by surplus Na-CM-cellulose. Therefore, it was possible to determine the total endo-1,4-β-glucanase activity of the total culture and to measure not only the endoglucanase activity found in the supernatant of the culture but also that adsorbed on residual Avicel. Figure 3 shows the formation of endo-1,4-β-glucanase activity of the total culture, determined by this method, during a 70-liter batch cultivation of *P. janthinellum* on 3% (wt/vol) Avicel as the sole carbon source.

Localization of endo-1,4-β-glucanases in cultures grown on cellulose. To study whether the endo-1,4-β-glucanases were bound to the cell wall or released into the culture medium, the endoglucanase activity produced during a 10-liter batch cultivation on 1.5% (wt/

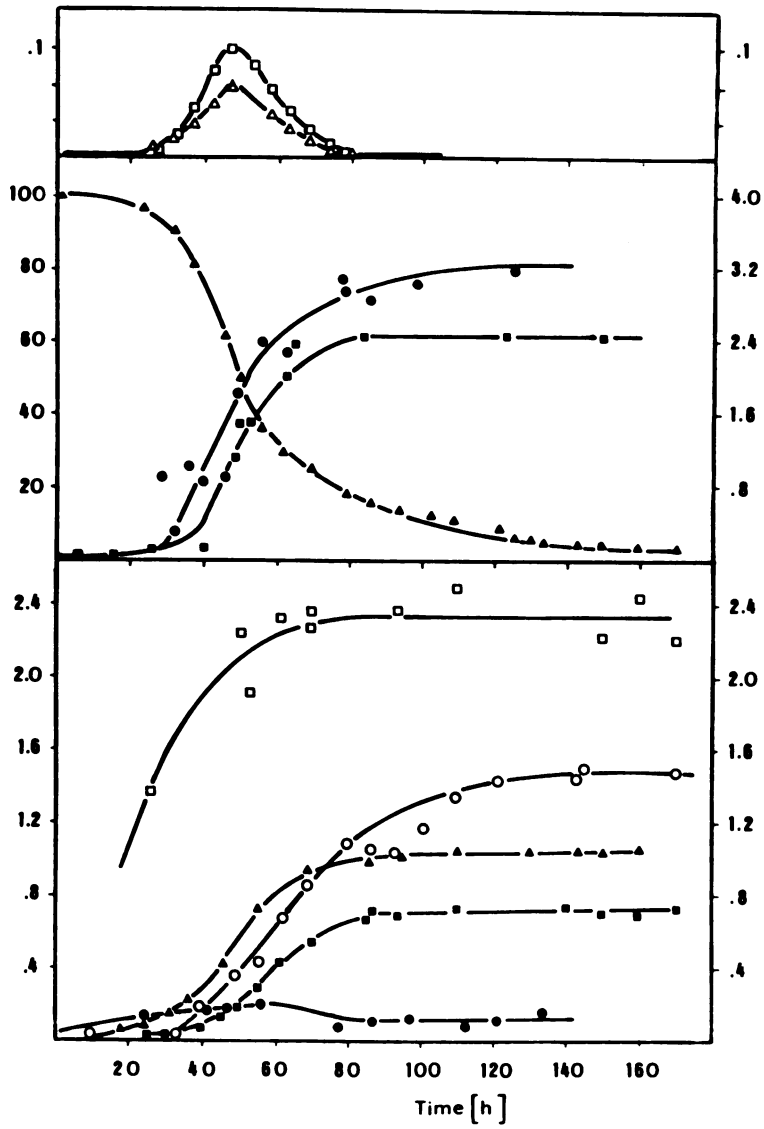

FIG. 1. *Growth and formation of 1,4-β-glucanases, 1,4-β-glucosidases, and proteases during a 70-liter batch cultivation on Avicel as the sole carbon source. Cultivation was carried out at 30°C and pH 5.0 in an 80-liter bioreactor containing 70 liters of basal medium with 3% (wt/vol) Avicel. It was aerated at 0.18 vol/vol per min and agitated with the intensor system (Giovanola Frères, S.A.) at 1,500 rpm. Symbols: (top) △, Q_{O_2}; □, Q_{CO_2} (both in grams per liter per hour). (Middle) ●, mycelial weight (grams per liter × 10^{-1}); ▲, cellulose concentration (percentage); ■, soluble protein (grams per liter). (Bottom) ▲, activity toward CM-cellulose; ■, activity toward Avicel; ○, cell-free 1,4-β-glucosidase activity; ●, cell-associated 1,4-β-glucosidase activity; □, protease activity (all in units per milliliter).*

vol) Na-CM-cellulose was measured in the total culture and supernatant by the rotational viscosimetric method. Over the whole period of cultivation, no differences in the endoglucanase activity of the total culture and supernatant could be detected. However, during cultivation of the fungus on 3% (wt/vol) Avicel, higher endoglucanase activities in the total culture than in the supernatant were measured up to 90 h after inoculation (Fig. 3). To determine whether the endoglucanases during this period were partially adsorbed on residual cellulose, bound to the cell wall, or both, desorption experiments were performed. Small samples of total culture and an excess of Na-CM-cellulose were incubated together with thorough mixing. After cen-

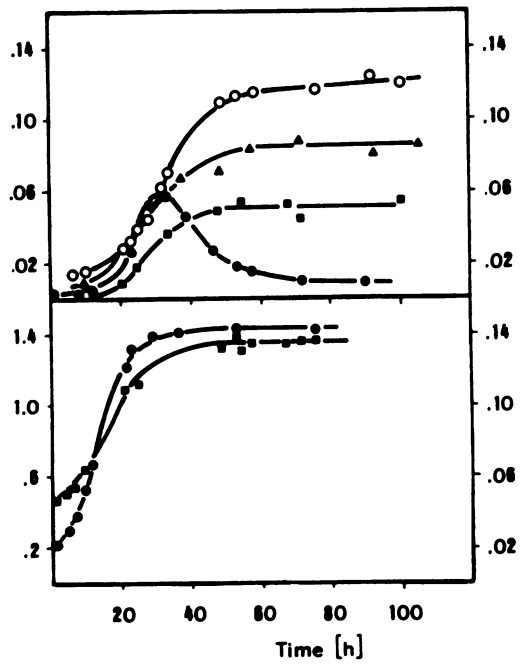

FIG. 2. *Growth and formation of 1,4-β-glucanases and 1,4-β-glucosidases during a 10-liter batch cultivation on Na-CM-cellulose as the sole carbon source. Cultivation was performed at 30°C and pH 5.0 in a 14-liter bioreactor containing 10 liters of basal medium with 1.5% (wt/vol) Na-CM-cellulose. It was aerated at 0.5 vol/vol per min and agitated with a turbine system containing two sets of six flat-bladed impellers at 400 rpm. Symbols: (top) ■, activity toward Avicel; ▲, activity toward CM-cellulose; ●, cell-associated 1,4-β-glucosidase activity; ○, cell-free 1,4-β-glucosidase activity (all in units per milliliter. (Bottom) ●, mycelial weight (grams per liter); ■, soluble protein (milligrams per liter).*

trifugation, the desorbed endoglucanase activity was determined by rotational viscosimetry (Fig. 3). The amounts of these desorbed endoglucanase activities were the same as the activity differences between the total culture and supernatant. Thus, we concluded that during cultivation of *P. janthinellum* on both Na-CM-cellulose and Avicel, the endo-1,4-β-glucanases were not bound to the surface of the mycelium.

Adsorption of endo-1,4-β-glucanases by Avicel. During the lag growth phase of a 70-liter batch culture of *P. janthinellum* on 3% (wt/vol) Avicel, 57% of the endoglucanase activity was adsorbed by cellulose (Fig. 3). This percentage decreased rapidly after 30 h together with the cellulose content as a consequence of the increasing glucanase activity. Ninety hours after inoculation, endoglucanases were found to be no longer adsorbed by residual Avicel, the concentration of which was lowered to 0.4% (wt/vol). Adsorption of the endoglucanases by 10% (wt/vol) Avicel was 80% from the beginning of the cultivation until the stationary growth phase, and after 130 h this adsorption capacity decreased very slightly (Fig. 3). Soluble protein and endo-1,4-β-glucanases were adsorbed by 10% (wt/vol) Avicel independently of the pH value within the range of 6.0 to 3.0, but the adsorption of soluble protein and endo-1,4-β-glucanases by 10% (wt/vol) Avicel decreased with increasing temperature (Table 1).

Growth and formation of 1,4-β-glucosidases on mono- and disaccharides. During growth of *P. janthinellum* on glucose, cellobiose, mannose, and maltose as the sole carbon sources, no measurable amounts of 1,4-β-glucanase activity could be detected. But cell-free and small amounts of cell-associated 1,4-β-glucosidase activity were always found (Fig. 4). The same phenomenon was observed in cultures grown on glycerol as the sole carbon source (P. Rapp, unpublished data). Even cultures grown on yeast extract, proteose peptone, Casamino Acids, and peptone from soybeans as the sole carbon sources produced considerable amounts of 1,4-β-glucosidase, but no measurable 1,4-β-glucanase activity (Grote, Ph.D. thesis, 1979). Table 2 summarizes the ratios of β-glucosidase activity to mycelial dry weight determined in cultures grown on Avicel, glucose, cellobiose, mannose, and maltose. They varied in the stationary growth phase from 0.14 to 0.22 U/mg. These results demonstrate the constitutive formation of 1,4-β-glucosidases by *P. janthinellum*.

Induction of endo-1,4-β-glucanase formation. The influence of cellobiose on the induction of endo-1,4-β-glucanase in 2.5% (wt/vol) Na-CM-cellulose medium with 8 g of wet mycelium per liter was studied. The uncommon sugar sophorose (2-β-glucopyranosyl-D-glucose), a powerful inducer of cellulase formation in *Trichoderma reesei* (15, 18, 21, 29), was also used.

For these investigations we used a rotational viscosimetric method similar to that of Eriksson and Hamp (10) (Fig. 5). An initial concentration of 4 mg of cellobiose per liter had a distinct additional inducing effect compared with the control with only Na-CM-cellulose as an inducing agent. This is illustrated in Fig. 5 by the difference in decrease of viscosity, which started earlier in the assays with additional cellobiose than in the control assay. Sophorose, on the other hand, did not exhibit any additional inducing effect compared with the control.

Location of 1,4-β-glucosidases. As mentioned above, 1,4-β-glucosidase activity was found both cell free and, to a small extent, associated with the mycelium. To investigate

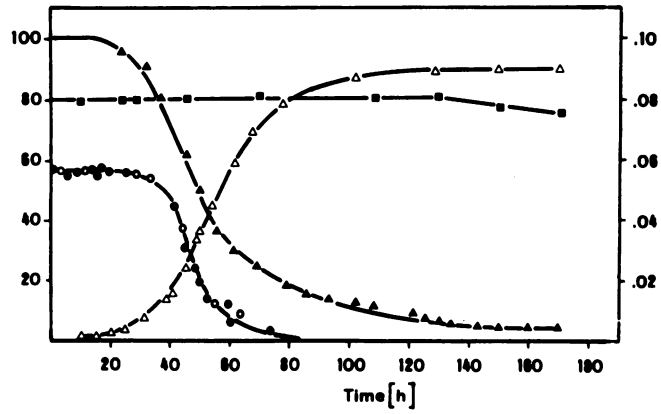

FIG. 3. *Formation of endo-1,4-β-glucanases and adsorption by residual and 10% (wt/vol) Avicel during a 70-liter batch cultivation on 3% (wt/vol) Avicel cellulose as the sole carbon source. Cultivation was performed as described in the legend to Fig. 1 and in the text. Symbols: Percentage of endo-1,4-β-glucanase activity (●) adsorbed by residual Avicel; (○) desorbed from residual Avicel; (■) readsorbed by an excess of 10% (wt/vol) Avicel. ▲, Cellulose concentration (percentage). △, Endo-1,4-β-glucanase activity in the total culture (relative units per milliliter).*

TABLE 1. *Adsorption of soluble protein and endo-1,4-β-glucanases by 10% (wt/vol) Avicel with respect to temperature*

Temp (°C)	mg of adsorbed soluble protein/g of Avicel	U of adsorbed endo-1,4-β-glucanase activity[a]/g of Avicel
20	0.148	0.020
30	0.144	0.018
40	0.122	0.017
50	0.121	0.016
60	0.093	0.013

[a] Determined by rotational viscosimetry.

the location and the strength of this association, mycelia free from excreted glucosidase activity were disrupted by sonication. No difference existed between the 1,4-β-glucosidase activity of intact mycelia and cell debris with glucose, cellobiose, and maltose as the carbon sources (Table 3). When Avicel was used as a substrate, the β-glucosidase activity of intact mycelium was a little higher than that of the cell debris. The reason for this difference may have been the cultivation time. In the decelerating growth phase when the mycelium grown on Avicel was harvested, β-glucosidases were still produced, and those which were in the status of excretion could be separated from the mycelium by sonication. In the late stationary growth phase, on the other hand, where the mycelia cultured on the three soluble sugars were harvested, no 1,4-β-glucosidases were excreted. Summarizing these observations, it seems that the small amounts of 1,4-β-glucosidase located at the surface of the mycelium were firmly associated with the cell wall of the fungus. The 1,4-β-glucosidase

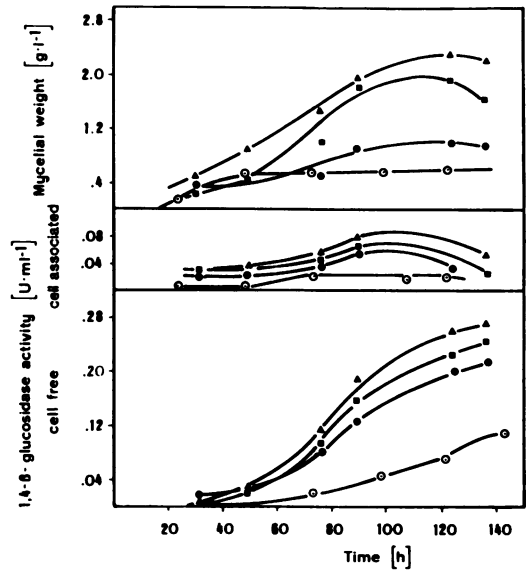

FIG. 4. *Growth and 1,4-β-glucosidase formation during 10-liter batch cultivations on (▲) cellobiose, (●) glucose, (■) maltose, and (⊙) mannose as sole carbon sources. Cultivation was carried out at 30°C and pH 5.0 in a baffled 14-liter bioreactor containing 10 liters of basal medium. A 0.1% (wt/vol) concentration of the corresponding carbon source was added daily. It was aerated at 0.5 vol/vol per min and agitated at 300 rpm with the turbine system described in Fig. 2.*

in the supernatants obtained after disruption of mycelia grown on Avicel, glucose, and cellobiose probably stemmed from the periplasmic space or was of true intracellular origin. They had 14

TABLE 2. *Formation of cell-free and cell-associated 1,4-β-glucosidase with respect to mycelial dry weight during cultivation on Avicel, cellobiose, glucose, mannose, and maltose*

Cultivation time (h)	1,4-β-Glucosidase activity (U/mg of mycelial dry wt)				
	Avicel[a]	Cellobiose[b]	Glucose[b]	Mannose[b]	Maltose[b]
76.0	0.16	0.11	0.18	0.10	0.11
99.5	0.18	0.14	0.19	0.16	0.12
123.5	0.19	0.14	0.22	0.22	0.16
136.0	0.19	0.15			0.16

[a] Cultivation was the same as described in Fig. 1.
[b] Cultivation was as described in Fig. 4. Enzyme activities were determined when the daily added sugars were consumed.

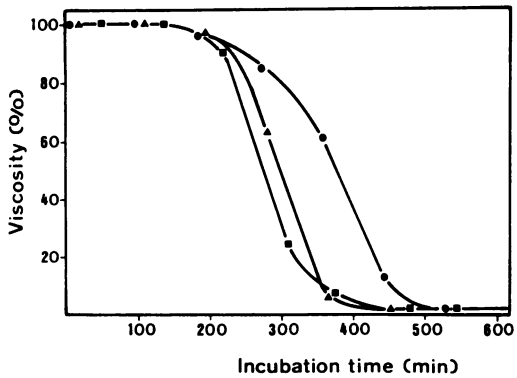

FIG. 5. *Influence of cellobiose on the induction of endo-1,4-β-glucanase formation in 2.5% (wt/vol) Na-CM-cellulose medium, determined by the rotational viscosimetric method. Mycelium concentration was 0.6 g/liter (determined as dried mycelium). Initial concentration of cellobiose:* (●) *0,* (▲) *4, or* (■) *30 mg/liter.*

to 25% of the activity measured in the crude extract (Table 3).

DISCUSSION

The rotational viscosimetric method, used in this paper for determination of endo-1,4-β-glucanase activity, is a variation of the capillary viscosimetric method developed by Almin and Eriksson (1, 2), Almin et al. (3), Eriksson and Hollmark (11), and Hulme (14). The use of a rotational instead of a capillary viscosimeter has the advantage of applying samples directly from the culture (broth and solids) and allows simple and rapid measurement of the total endo-1,4-β-glucanase activity, including that bound to the surface of the mycelium and adsorbed on residual Avicel. A prerequisite for determination of the total endoglucanase activity in the culture

TABLE 3. *Location of 1,4-β-glucosidases in mycelia from cultures grown on Avicel, cellobiose, glucose, and maltose*

Substrate	1,4-β-Glucosidase activity (%) of:		
	Intact mycelium[a]	Cell debris[b]	Supernatant after cell disruption[a]
Avicel[c]	91	75	25
Cellobiose[d]	82	80	20
Glucose[d]	86	86	14
Maltose[d]	100	100	0

[a] Expressed as percentage of the crude extract activity obtained after cell disruption.
[b] Calculated from the differences of activities in the crude extract obtained after cell disruption and the supernatant and expressed as percentage of the crude extract activity.
[c] Cultivation was performed in 1-liter shake flasks for 120 h.
[d] Cultivation was as described in Fig. 4. Mycelia were harvested 136 h after inoculation.

(broth and solids) is, of course, the complete desorption of those endoglucanases adsorbed on residual cellulose during the viscosimetric measurement. This is the case for the endo-1,4-β-glucanases from *P. janthinellum*. They were rapidly and completely desorbed, probably due to the competition of surplus Na-CM-cellulose with residual Avicel as a substrate for the endoglucanases. Therefore, a short reaction time for the viscosimetric determination can be used.

The amount of endo-1,4-β-glucanases from *P. janthinellum* adsorbed by residual Avicel seems to depend on the cellulose concentration. At the beginning of the cultivation on 3% (wt/vol) Avicel, 57% of the endoglucanase activity was adsorbed by cellulose. This percentage declined during the cultivation, when the glucanase activity increased and the cellulose content decreased. Thus, almost no endo-1,4-β-glucanases were adsorbed by residual cellulose when the Avicel content was lowered to 0.6% (wt/vol). After addition of 10% (wt/vol) Avicel, 80% of the endoglucanases were readsorbed (Fig. 3). These percentages agree approximately with those for the cellulase from *T. viride* (17).

The adsorption of endoglucanases and soluble protein from *P. janthinellum* by Avicel decreased with increasing temperature but was largely independent of the pH value. These results are comparable to those of *T. viride* cellulases (24).

The solid and rigid nature of most cellulosic substrates raises the question of whether cell-bound or cell-free enzymes catalyze the cellulose hydrolysis. For the intimate contact necessary for the formation of an enzyme-substrate com-

plex, cell-free glucanases seem to be more suitable than cell-bound ones when an insoluble cellulose is used as the substrate. During cultivation of *P. janthinellum*, not only on Avicel but also on Na-CM-cellulose, no measurable amount of cell-bound endo-1,4-β-glucanase activity could be detected. Eriksson and Hamp (10) obtained similar results with *Sporotrichum pulverulentum* (identical to *Phanerochaete chrysosporium*), which released the endoglucanases produced on CM-cellulose into the medium, although they appeared on the fungal cell wall 2 h before they could be traced in the medium. The results concerning the location of cellulases from *T. viride* are contradictory. On the one hand, Berg and Pettersson (6) reported that much of the *T. viride* cellulase is cell bound during active growth and released into the medium only when cellulose is consumed and the fungus is starving. Similar observations were made by Eriksson and Hamp (10). They found that none of the endoglucanases from *T. viride*, induced by sophorose, could be detected extracellularly. On the other hand, Sternberg and Mandels (29) discovered that the *T. viride* cellulases induced by sophorose were not appreciably associated with the mycelium before the enzymes appeared in the medium, indicating that synthesis and secretion of cellulases are closely connected. Similarly, Vaheri et al. (32) found that cellulases of *T. reesei* QM 9414 induced by cellobiose were actively released into the medium even in the early stages of cultivation.

Induction of endo-1,4-β-glucanase formation by cellobiose and sophorose has been studied extensively for *T. viride* (10, 15, 18, 19, 21, 22, 29, 32). In this fungus, sophorose is a much more powerful inducer of endoglucanase formation than cellobiose, whereas cellobiose induces this formation in *S. pulverulentum* (*P. chrysosporium*) better than sophorose (10). On the contrary, the endo-1,4-β-glucanase formation of *P. janthinellum* is not induced by sophorose but by cellobiose.

In cultures of *P. janthinellum* grown on celluloses and mono- and disaccharides, 1,4-β-glucosidases were predominantly cell free, with only a very small amount associated with the mycelium. 1,4-β-Glucosidase from *T. reesei*, on the contrary, was essentially cell bound and not released unless the cells were autolyzing (6, 32). In the case of *S. pulverulentum* (*P. chrysosporium*), only cell-wall bound β-glucosidases were formed with cellobiose as the sole carbon source, whereas for the extracellular excretion cellulose seemed to be a necessary carbon source (9). The cell-free and cell-associated 1,4-β-glucosidases from *P. janthinellum* are formed constitutively and differ on this point from those of *S. pulverulentum* (*P. chrysosporium*) (13) and *T. reesei* (15), the formation of which is inducible. Similar to *P. chrysosporium* (28), *P. janthinellum* produces not only cell-associated and cell-free but also soluble intracellular 1,4-β-glucosidase. The exact location, however, of this soluble, intracellular β-glucosidase was not determined, and it also not known whether the extra- and intracellular, as well as the glucosidases tightly associated with the cell wall, have the same properties.

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