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Phanerochaete chrysosporium β-Glucosidases: Induction, Cellular Localization, and Physical Characterization

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Phanerochaete chrysosporium produces intracellular soluble and particulate β-glucosidases and an extracellular β-glucosidase. The extracellular enzyme is induced by cellulose but repressed in the presence of glucose. The molecular weight of this enzyme is 90,000. The K_m for *p*-nitrophenyl-β-glucoside is 1.6×10^{-4} M; the K_i for glucose, a competitive inhibitor, is 5.0×10^{-4} M. The K_m for cellobiose is 5.3×10^{-4} M. The intracellular soluble enzyme is induced by cellobiose; this induction is prevented by cycloheximide. The presence of 300 mM glucose in the medium, however, had no effect on induction. The K_m for *p*-nitrophenyl-β-glucoside is 1.1×10^{-4} M. The molecular weight of this enzyme is ~410,000. Both enzymes have an optimal temperature of 45°C and an E_{act} of 9.15 kcal (ca. 3.83×10^4 J). The pH optima, however, were ~7.0 and 5.5 for the intracellular and extracellular enzymes, respectively.

White rot is characterized by the breakdown of the lignin, hemicellulosic, and cellulosic components of wood. The fungi responsible for white rot, including *Phanerochaete chrysosporium* (4), have potential utility in such diverse applications as wood pulping and the production of chemicals from lignocellulosic waste. *P. chrysosporium* has been the subject of numerous investigations concerning lignin degradation (1, 11, 14) and cellulose degradation (6).

The extracellular cellulolytic enzyme system of the closely related fungus *Sporotrichum pulverulentum* has been shown to consist of endo-1,4-β-glucanases, an exo-1,4-β-glucanase, and a β-glucosidase (6). Until recently, little information on the induction or molecular properties of the β-glucosidases (E.C. 3.2.1.21) has been available. In this paper we describe studies on the cellular localization, the induction, and the catabolite repression, as well as the kinetics and physical characteristics, of this enzyme from *P. chrysosporium*. Studies on β-glucosidases from several other basidiomycetes have been reported (5, 10, 19).

MATERIALS AND METHODS

Media and reagents. Growth media were obtained from Difco. Microcrystalline cellulose was from Baker Chemical Co. BioGel A-1.5m was purchased from BioRad. All other reagents were obtained from Sigma.

Growth of mycelia. A culture of *P. chrysosporium* ME446, obtained from the U.S. Forest Products Laboratory, Madison, Wis., was maintained on slants of Vogel medium N (18), with thiamine replacing biotin (modified Vogel), and supplemented with 3% malt extract and 0.25% yeast extract.

Conidia were washed from slants, filtered through glass wool, and diluted with distilled water to a concentration of 10^5/ml of medium. Cells were grown in Erlenmeyer flasks on a New Brunswick G-10 shaker operating at a speed of 150 rpm and describing a 2-inch (ca. 5-cm) circle. The cells were grown at 28°C, in a medium consisting of modified Vogel salts and 2% glucose, or other carbon sources where indicated. The mono- and disaccharide carbon sources were autoclaved separately and added immediately before inoculation.

Intracellular enzyme induction and preparation. Cells grown for 48 h in submerged culture containing 2% glucose were aseptically harvested by suction filtration, washed, and transferred to modified Vogel medium supplemented with an appropriate carbon source for enzyme induction. Samples of the mycelial suspension were then removed periodically, washed by suction filtration, and frozen. Frozen mycelium was ground with sand in a chilled mortar. All subsequent steps were performed at 0 to 4°C. The broken cells were extracted for 5 min in distilled water and centrifuged at 27,000 × *g* for 30 min. The supernatant was assayed for soluble enzyme activity and protein concentration. The supernatants from maximally induced cells were pooled, and solid ammonium sulfate (Mann, enzyme grade) was added to 90% saturation. The solution was stirred for 30 min and centrifuged at 27,000 × *g* for 20 min. The precipitate was suspended in distilled water, dialyzed until sulfate free, and lyophilized. The cell pellet was washed 10 to 15 times with distilled water until the final wash showed no enzyme activity, and then the pellet was assayed for particulate enzyme activity and protein concentration.

Extracellular enzyme induction and preparation. Cells were grown in 1 liter of modified Vogel medium containing either 0.25% cotton or microcrystalline cellulose as the carbon source. Enzyme produc-

tion was monitored by assaying 0.90 ml of culture fluid for activity. After 10 to 12 days, the culture fluid was filtered, and the filtrate was cooled to 0°C. Solid ammonium sulfate was added to bring the solution to 90% saturation. The solution was stirred for 30 min and centrifuged at 27,000 × *g* for 20 min. The precipitate was suspended in distilled water, dialyzed until sulfate free, and lyophilized.

Assays. Intracellular soluble and insoluble *p*-nitrophenyl-β-glucosidase (PNPGase) activity was measured in a 1-ml reaction mixture containing 50 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) buffer and 5 mM *p*-nitrophenyl-β-glucoside (PNPG). Reactions were started by the addition of 50 to 900 μl of enzyme and were carried out at 45°C for 10 min. After the reaction, 0.5 ml of 1 M NaOH in 1 M tris(hydroxymethyl)aminomethane buffer was added to develop the color, and the tubes were cooled on ice. The *p*-nitrophenol produced was measured at 400 nm and compared to a standard curve.

Extracellular enzyme was assayed in a similar manner except that 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.5) was used as the buffer.

Cellobiase activity was measured by a modification of the invertase assay of Gascon et al. (8), substituting 250 mM cellobiose for sucrose. The assay was performed on enzyme preparations which had been dialyzed free of glucose. Protein concentration was measured by the method of Lowry et al. (13), using bovine serum albumin as the standard. Glucose concentrations were determined by use of a glucose oxidase procedure (8).

Gel filtration. Ammonium sulfate-fractionated, dialyzed, and lyophilized enzymes were dissolved in distilled water and gel filtered at 4°C on a column of BioGel A-1.5m (1.5 by 52 cm) equilibrated in 20 mM tris(hydroxymethyl)aminomethane-chloride (pH 7.4) containing 100 mM NaCl. Fractions (1.5 ml) were collected, and samples were examined for enzyme activity.

RESULTS

The production of three intracellular enzyme activities is shown in Fig. 1. When pregrown cells were transferred to a medium containing cellobiose, intracellular soluble PNPGase and cellobiase activities were induced in a similar fashion, reaching a maximum approximately 20 h after the introduction of cellobiose. Insoluble PNPGase activity increased more slowly, reaching a maximum after approximately 33 h. Particulate cellobiase activity could not be detected within this time period. The presence of glucose in 0 to 300 mM concentration had a negligible effect on the induction by 1% cellobiose of the intracellular soluble enzyme.

The time course of formation of the extracellular β-glucosidase is shown in Fig. 2. Cells grown in the presence of cellulose produced maximal activity after approximately 7 days. When the cells were grown in the presence of 0.25% cellulose and 1% glucose, extracellular β-glucosidase did not appear until day 9. There was a 4-day lag between the disappearance of the exogenous glucose in the medium and the appearance of β-glucosidase activity. With 3% glucose in the medium, β-glucosidase did not appear. These results indicate that glucose or glucose metabolite(s) represses the formation of the extracellular β-glucosidase but not the formation of intracellular β-glucosidase. The mechanism of this repression is not known.

The capability of various substrates to induce intracellular and extracellular β-glucosidase is

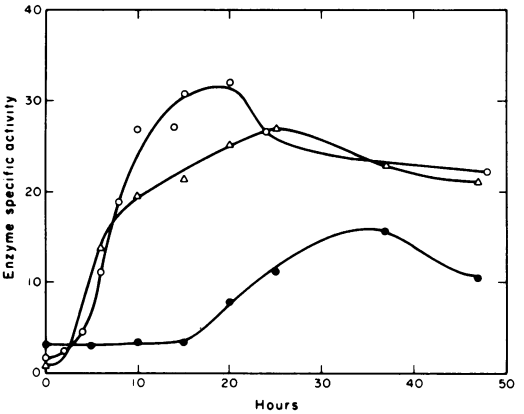

FIG. 1. *Development of three β-glucosidase activities. Enzyme specific activity is expressed as (micromoles of p-nitrophenol released per minute per milligram of protein) × 10^3 for intracellular soluble β-glucosidase* (○) *and insoluble β-glucosidase* (●) *and as (micromoles of glucose released per minute per milligram of protein) × 10^2 for intracellular soluble cellobiose* (△).

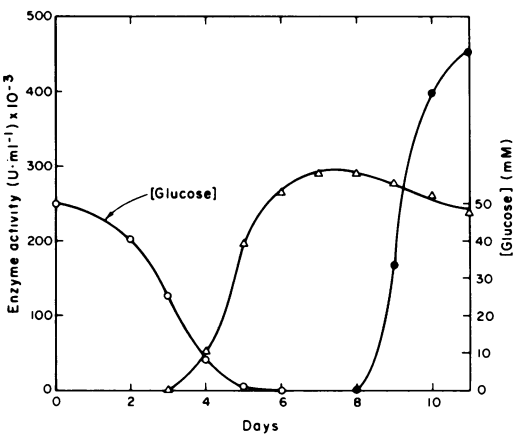

FIG. 2. *Production of extracellular β-glucosidase and its repression by glucose. Conidia were inoculated into flasks containing cotton in modified Vogel medium with* (●) *or without* (△) *glucose. When glucose was present initially, it was consumed rapidly* (○).

shown in Table 1. The strongest inducers of the extracellular β-glucosidase were cellulosic polymers, whereas cellobiose was a very poor inducer of this form of the enzyme. Intracellular β-glucosidase was induced by growth on a variety of substrates, although in this case cellobiose induced maximal activity (27.5 units/mg of protein).

The addition of cycloheximide (2.0×10^{-5} M) to pregrown cells during the appearance of intracellular β-glucosidase halted any further increase in enzyme activity (Fig. 3). This result suggests that intracellular β-glucosidase is synthesized de novo when cellobiose is introduced as the sole source of carbon.

Gel filtration of the intracellular and extracellular β-glucosidases indicated that they also differed with respect to molecular weight (Fig. 4). Whereas the intracellular enzyme eluted as a peak of molecular weight 410,000, the extracellular enzyme eluted as a single symmetrical peak with a molecular weight of 90,000 as calculated by the method of Andrews (2) (Fig. 4, insert).

PNPG assays with both forms of the enzyme were linear with respect to time and protein concentration. The pH optimum for the intracellular enzyme was 7.0, whereas the pH optimum for the extracellular enzyme was 5.5. The temperature optimum for both enzymes was approximately 45°C. When the data were plotted according to Arrhenius, straight lines were obtained. From this the energy of activation for both enzymes was calculated to be 9.15 kcal (ca. 3.83×10^4 J). PNPG saturation curves for both enzymes were hyperbolic. From Lineweaver-Burke plots, K_m values of 1.1×10^{-4} M and 1.6×10^{-4} M were calculated for the intracellular and extracellular β-glucosidases, respectively. The cellobiose saturation curve for the extracellular enzyme was hyperbolic, and the K_m for cellobiose was calculated to be 5.3×10^{-4} M. Figure 5 shows a Dixon plot using extracellular β-glucosidase. The plot indicates that glucose is a competitive inhibitor with respect to PNPG, probably binding at the active site. The K_i for glucose was calculated to be 5.0×10^{-4} M.

TABLE 1. *Inducing ability of various carbon sources for the intracellular and extracellular enzymes*

Carbon source[a]	Intracellular β-glucosidase[a] sp act (μmol/min per mg of protein)	Extracellular β-glucosidase[b] (U/ml) × 10^3
Cellobiose	27.5	0.5
Cotton	12.4	9.8
Walseth cellulose	11.8	3.2
Microcrystalline cellulose	11.3	14.9
Phenyl-β-D-glucoside	11.0	
Sucrose	8.9	
Calcium cellobionate	7.6	
myo-Inositol	7.3	
Xylan	5.8	
Glycerol	5.5	
Xylose	5.0	
Galactose	3.4	
Maltose	3.3	
No carbon	3.0	
Spruce woodmeal	2.1	
Mannose	1.1	
Mannitol	0.8	
Fructose	0.7	
Glucose	0.5	

[a] Soluble carbon sources are in 1% concentration. Insoluble carbon sources are present at 0.25 g per 100 ml.

[b] Conidia were inoculated into flasks containing the indicated carbon sources. A 0.90-ml sample of filtered culture fluid was assayed for enzyme activity as described in the text.

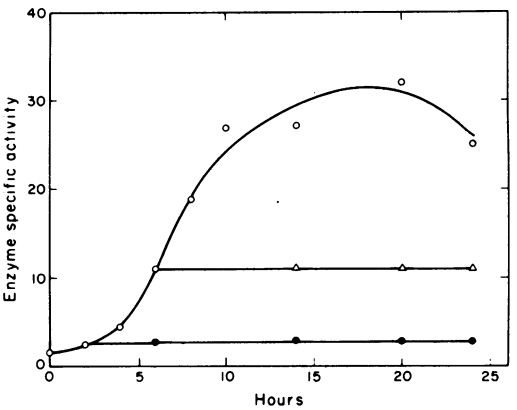

FIG. 3. *Inhibition of the synthesis of intracellular β-glucosidase by cycloheximide. Normal production of enzyme induced with cellobiose* (○) *is completely prevented upon the addition of cycloheximide* (2×10^{-5} *M) at 2* (●) *or 6* (△) *h after the addition of cellobiose.*

DISCUSSION

It is clear that several forms of β-glucosidase are produced by *P. chrysosporium*. The relative contribution of each enzyme to the hydrolysis of cellulose is unknown. The evolutionary advantage for the existence of several enzymes is also unclear. It has been suggested that intracellular β-glucosidases have important functions in developmental processes in higher fungi, given the high concentration of β-glucans in the cell walls of various fungi (19). The soluble internal β-glucosidase is most likely involved primarily with energy-assimilating functions, however,

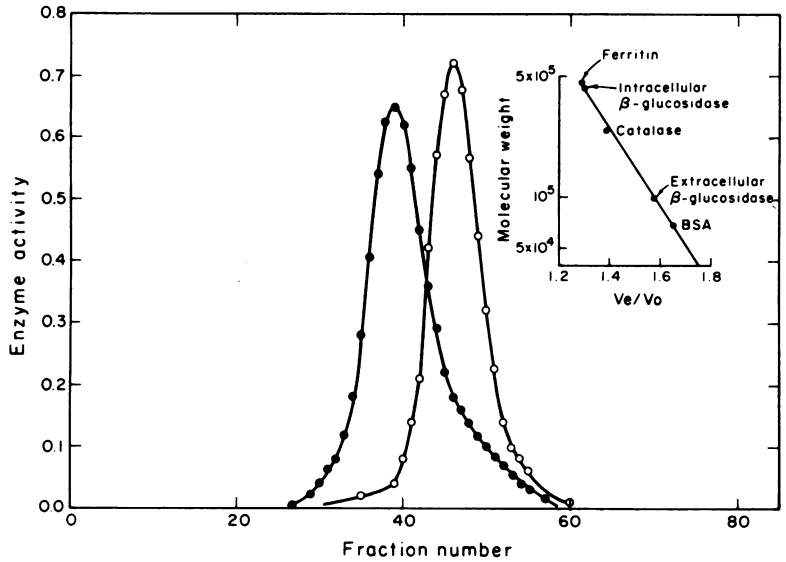

FIG. 4. *BioGel A-1.5m gel filtration of the intracellular (●) and extracellular (○) β-glucosidases. A 2-ml sample of enzyme was applied to a column (1.5 by 52 cm) of BioGel A-1.5m equilibrated with 20 mM tris(hydroxymethyl)aminomethane-chloride in 100 mM NaCl (pH 7.4). Insert, log molecular weight versus V_e/V_0 plot indicates that the molecular weight of the extracellular enzyme is 90,000, and that of the intracellular enzyme is 410,000.*

since it is rapidly induced by cellobiose. The external enzyme along with other cellulolytic enzymes is produced in the presence of cellulose. Since this organism is most often found in decaying wood, there may be some advantage to the organism in maintaining some small level of extracellular glucose. Green (9) and Koenigs (12) have suggested lignolytic and cellulolytic mechanisms involving an external glucose oxidase requiring a supply of glucose. Eriksson has described a similar scheme involving cellobiose and cellobiose:quinone oxidoreductase for the degradation of lignin (6). It is not unlikely that all these systems operate, since cellobiose and glucose are both produced when cellulose is degraded by extracellular enzymes recovered from *Sporotrichum* (7). The extracellular glucose and cellobiose levels may be important in the regulation of cellulolytic enzymes so that control of these levels may be a component in the overall control of lignocellulose degradation.

In a recent paper, which appeared while this study was being completed, Deshpande et al. (5) examined the extracellular and cell wall-bound β-glucosidases in *Sporotrichum pulverulentum*. These authors also found that the extracellular enzyme was induced by cellulose and claim that cellobiose induces a cell wall-bound enzyme. They did not assay for soluble intracellular β-glucosidase activity in cell-free preparations. In *P. chrysosporium* this soluble intracellular enzyme represents approximately 90 and 75% of the total β-glucosidase activity in cells induced with cellobiose for 10 and 25 h, respectively.

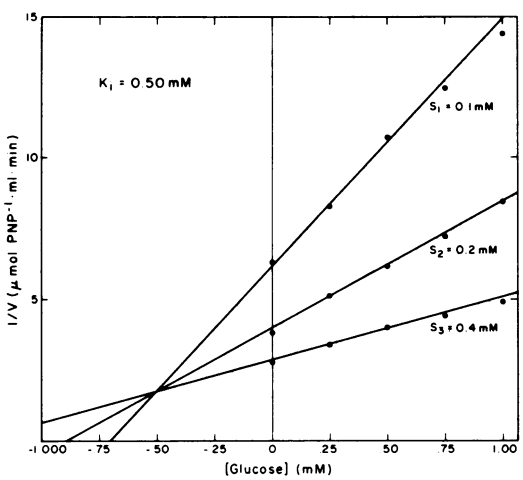

FIG. 5. *Competitive inhibition by glucose of extracellular β-glucosidase activity. Dixon plot of the effect of glucose on the hydrolysis of PNPG. S_1, S_2, and S_3 refer to the concentration of PNPG in the reaction mixture. PNP, p-Nitrophenol.*

The molecular weight of the *P. chrysosporium* extracellular enzyme as shown by gel filtration is 90,000. This is different from the weights of enzymes from *Trichoderma viridiae* (47,000)

(3), from *Lenzites trabea* (320,000) (10), and from *S. pulverulentum* (~170,000) (5). The K_m values for PNPG of the *P. chrysosporium* enzymes (~10^{-4} M) are similar to those determined for the enzymes from *L. trabea* (10), from *Saccharomyces lactis* (17), and from the extracellular fluid of *S. pulverulentum*, but are 30-fold lower than the K_m for the intracellular *Neurospora crassa* aryl-β-glucosidase (15).

The inhibition of the extracellular enzyme by glucose was competitive, in contrast to the noncompetitive inhibition by glucose of the *L. trabea* enzyme (10).

Although this work suggests that the intracellular and extracellular enzymes are products of different structural genes, the possibility exists that the large intracellular enzyme is actually a precursor of the extracellular enzyme, and that the different induction kinetics are due to postsynthetic processing and secretion. We are currently pursuing genetic and further biochemical studies of these enzymes with respect to these questions.

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