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Purification and Characterization of a Cellulose-Binding β-Glucosidase from Cellulose-Degrading Cultures of *Phanerochaete chrysosporium*

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Extracellular β-glucosidase from cellulose-degrading cultures of *Phanerochaete chrysosporium* was purified by DEAE-Sephadex chromatography, by Sephacryl S-200 chromatography, and by fast protein liquid chromatography (FPLC) using a Mono Q anion-exchange column. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis of FPLC-purified β-glucosidase indicated the presence of three enzyme forms with molecular weights of 96,000, 98,000, and 114,000. On further fractionation with a microcrystalline cellulose column, the 114,000-molecular-weight β-glucosidase, which had 82% of the β-glucosidase activity, was bound to cellulose. The β-glucosidases with molecular weights of 96,000 and 98,000 did not bind to cellulose. The cellulose-bound β-glucosidase was eluted completely from the cellulose matrix with water. Cellulose-bound β-glucosidase catalyzed *p*-nitrophenylglucoside hydrolysis, suggesting that the catalytic site is not involved in cellulose binding. When the cellulose-binding form was incubated with papain for 20 h, no decrease in the enzyme activity was observed; however, approximately 74% of the papain-treated glucosidase did not bind to microcrystalline cellulose. SDS-PAGE analysis of the nonbinding glucosidase produced by papain indicated the presence of three bands with molecular weights in the range of 95,000 to 97,000. On the basis of these results, we propose that the low-molecular-weight (96,000 and 98,000) non-cellulose-binding β-glucosidase forms are most probably formed from the higher-molecular-weight (114,000) cellulose-binding β-glucosidase via extracellular proteolytic hydrolysis. Also, it appears that the extracellular β-glucosidase from *P. chrysosporium* might be organized into two domains, a cellulose-binding domain and a catalytic domain. Kinetic characterization of the cellulose-binding form is also presented.

Phanerochaete chrysosporium, a white rot basidiomycete, degrades wood completely (15). Since cellulose constitutes 40 to 60% of all plant woody tissues, its degradation by *P. chrysosporium* is of interest. The fungal cellulolytic system consists of an endoglucanase, an exocellobiohydrolase, and a β-glucosidase. Endoglucanase hydrolyzes the internal glycosidic linkages of cellulose, exocellobiohydrolase hydrolyzes cellobiose from the nonreducing ends of the cellulose polymer, and β-glucosidase cleaves cellobiose into two glucose units (8). β-Glucosidase does not act upon cellulose directly; nevertheless, it is important because cellobiose is a competitive inhibitor of cellulases and β-glucosidase reduces this inhibition by hydrolyzing cellobiose to glucose (27). Cellulases with low levels of β-glucosidase hydrolyze cellulose slowly, and in such cases the addition of β-glucosidase enhances cellulose hydrolysis (18, 28). In addition to cellulases, cellulose-degrading cultures of *P. chrysosporium* produce the following two cellobiose-oxidizing enzymes: cellobiose dehydrogenase, a hemoflavoenzyme (2, 5), and cellobiose:quinone oxidoreductase, a flavoenzyme (29). The cellobiose-oxidizing enzymes oxidize cellobiose to cellobionolactone in the presence of electron acceptors such as ferricytochrome *c*, dichlorophenol-indophenol, and quinones (2, 5). Bao and Renganathan (4) demonstrated that cellobiose dehydrogenase enhances microcrystalline cellulose hydrolysis by cellulases.

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P. chrysosporium produces three different β-glucosidases—extracellular, intracellular, and cell wall bound—depending on the carbon source (10, 24). Cellulose induces extracellular and intracellular β-glucosidases (10), whereas cellobiose induces intracellular and cell wall-bound enzymes (24). Deshpande et al. (10) purified five isozymes of extracellular β-glucosidase from cellulose-degrading cultures of *P. chrysosporium* (*Sporotrichum pulverulentum*), and the molecular weights of these isozymes ranged from 165,000 to 182,000. Smith and Gold (24) partially purified an extracellular β-glucosidase from *P. chrysosporium* ME-446 and characterized it as a monomer with a molecular weight of 90,000. Recently we optimized the culture conditions for the production of cellulose-degrading enzymes by *P. chrysosporium* (3). Succinate cultures adjusted to an initial pH of 4.5 and with cotton linters as the carbon source produced high levels of β-glucosidase (3). In the present study, an extracellular β-glucosidase from succinate cultures of *P. chrysosporium* was purified and characterized; also, this β-glucosidase was demonstrated to bind to microcrystalline cellulose, a characteristic generally associated only with cellulases (14).

MATERIALS AND METHODS

Organism. Stock cultures of *P. chrysosporium* OGC101 were maintained on slants of Vogel medium N supplemented with 3% malt extract and 0.25% yeast extract (1).

Culture conditions. Stock cultures of *P. chrysosporium* were inoculated into a medium (20 ml) supplemented with 2% glucose and 0.5% yeast extract. The cultures were incubated at 37°C for 72 h without shaking. Four 72-h cultures were homogenized in a blender for 20 s and inoculated into 1 liter of medium containing 25 mM succinate, 20 mM ammonium phosphate, yeast extract (100 mg/ml), ferric nitrate (15 mg/ml), thiamine hydrochloride (0.1 mg/ml), and other

TABLE 1. Summary of β-glucosidase purification

Purification step	Amt of protein (mg)	Total activity (U)	Sp act (U/mg)[a]	Yield (%)	Purification (fold)
Culture filtrate	1,650	420.0	0.254	100	1
Ultrafiltration	1,352	400.0	0.3	95.0	1.18
DEAE–Sephadex A-50 chromatography I	284	363.0	1.3	86.0	5.12
DEAE–Sephadex A-50 chromatography II	172	313.0	1.8	74.0	7.08
Sephacryl S-200 chromatography	21.0	236.0	11.2	56.0	44.09
FPLC I	2.4	63.0	26.3	15.0	103.5
FPLC II	0.95	32.0	33.7	7.6	132.7
Microcrystalline cellulose column					
Cellulose-binding form	0.8	26.4	33.0	6.1	129.9
Non-cellulose-binding form	0.2	6.6	33.0	1.5	129.9

[a] β-Glucosidase activity was determined by monitoring the release of *p*-nitrophenol from PNPG at 400 nm.

mineral salts as described previously (2, 3). The initial pH of the medium was adjusted to 4.5. Cotton linters (10 g/liter) served as the carbon source. The cultures were incubated at 28°C on a rotary shaker (150 rpm).

β-Glucosidase purification. The extracellular medium (2 liters) from 10- to 12-day-old *P. chrysosporium* cultures was filtered through glass wool to remove mycelial fragments. The filtered medium was centrifuged to remove particulates. EDTA (5 mM) and phenylmethylsulfonyl fluoride (0.5 mM) were added to inhibit extracellular proteases. The medium pH was adjusted to 7.5, and the medium was concentrated to 10% of its initial volume by ultrafiltration using a hollow fiber cartridge (Type HIP10-20; Amicon, Beverly, Mass.) and dialyzed against 50 mM phosphate, pH 7.5. The dialyzed proteins were applied to a DEAE-Sephadex column (10 by 4 cm) equilibrated with 50 mM phosphate, pH 7.5. β-Glucosidase did not bind to this column. The unbound proteins were concentrated and loaded on another DEAE-Sephadex column (30 by 2.5 cm) equilibrated with 50 mM phosphate, pH 7.5. Unbound proteins which contained β-glucosidase were fractionated on a Sephacryl S-200 column (60 by 2.5 cm) equilibrated with 50 mM phosphate, pH 6. Fractions containing β-glucosidase activity were pooled, concentrated, and further purified twice by fast protein liquid chromatography (FPLC) using a Mono Q HR 5/5 column (Pharmacia Fine Chemicals, Piscataway, N.J.). FPLC separations were performed in 10 mM Tris-HCl, pH 8, with a 1 M NaCl gradient. In the final step, β-glucosidase was purified to homogeneity by microcrystalline cellulose (Sigmacell Type-50) affinity chromatography. β-Glucosidase was loaded on a cellulose column (8 by 0.6 cm) equilibrated with 50 mM phosphate, pH 6. The unbound proteins were washed off the column, and the bound β-glucosidase was removed by elution with double-distilled and deionized water.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 10% gel according to the method of Laemmli (19) by using a Mini-Protean system (Bio-Rad Laboratories, Richmond, Calif.). The gel was stained with 0.1% Coomassie brilliant blue in a solution containing 50% methanol and 15% acetic acid.

Enzyme assays. β-Glucosidase activity was determined by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl-β-glucoside (PNPG). The enzyme solution (5 to 50 μl) was incubated with 1 mM PNPG in 1 ml of 50 mM phosphate, pH 4.5, at room temperature for 5 min. The enzyme reaction was stopped by the addition of 0.2 ml of 1 M sodium carbonate, and the color developed because of the *p*-nitrophenolate anion was monitored at 400 nm (ε = 18.3 mM^{-1} cm^{-1}). One unit of β-glucosidase activity is defined as that amount of enzyme which will hydrolyze 1 μmol of PNPG per min.

β-Glucosidase activity towards cellobiose and cello-oligosaccharides was determined by assaying the amount of glucose released by the glucose oxidase-peroxidase method (16). β-Glucosidase (5 to 50 μl) was incubated with a substrate in 3 ml of 50 mM sodium acetate, pH 5. Aliquots (0.5 ml) were withdrawn at regular intervals, and the reaction was arrested by the addition of 50 mM Tris-HCl, pH 9 (0.48 ml). Tris buffer addition shifted the pH to 8, and at this pH β-glucosidase was inactive. *o*-Dianisidine (10 mM, 10 μl), glucose oxidase (5 U), and horseradish peroxidase (2.5 U) were added to the enzyme reaction mixture, and it was incubated at room temperature for 30 min. Then 1 drop of concentrated sulfuric acid was added to stabilize the color, and the A_{420} was determined. The amount of glucose produced was calculated from a standard curve for glucose.

Protein and carbohydrate measurements. Protein concentrations were determined by the bicinchoninic acid method (25). The neutral carbohydrate content of the β-glucosidase was determined by the phenol-sulfuric acid method (11). The amount of sugar was calculated from a standard curve for glucose.

Effect of pH on β-glucosidase stability. β-Glucosidase stability was examined at pH 2 to 10. A fresh sample (0.55 U) was incubated in an appropriate buffer (1 ml, 50 mM) at room temperature for 20 h. The activity remaining was determined by PNPG assay. Buffers used in this experiment were as follows: KCl-HCl, pH 2; glycine-HCl, pH 3; sodium acetate, pH 4 and 5; potassium phosphate, pH 6 and 7; Tris-HCl, pH 8 and 9; and glycine-sodium hydroxide, pH 10.

Effect of temperature on β-glucosidase stability. Stability against heat inactivation was determined by incubating a fresh sample of β-glucosidase (0.24 U) at various temperatures (45 to 75°C) in 50 mM phosphate, pH 4.5. Aliquots were withdrawn at regular intervals and cooled in an ice bath, and the remaining activity was estimated by the PNPG assay.

Effect of pH on β-glucosidase activity. Enzyme activity was determined in the following buffer systems (50 mM): acetate, pH 4 to 8, and phosphate, pH 3 to 9.

PNPG hydrolysis by cellulose-immobilized β-glucosidase. β-Glucosidase (0.06 U) was immobilized on microcrystalline cellulose by being passed through a cellulose column equilibrated with 50 mM phosphate, pH 4.5; unbound proteins were removed by elution with buffer. The cellulose was suspended in 7 ml of 50 mM phosphate, pH 4.5, and PNPG was added to a final concentration of 1 mM. Samples (0.2 ml) were taken every 5 min, and the reaction was arrested by the addition of 0.2 M sodium carbonate. The samples were centrifuged, and the A_{400} of the supernatants was determined.

Cellobionolactone hydrolysis by purified β-glucosidase and by extracellular proteins. The extent of cellobionolactone hydrolysis was determined by monitoring glucose release. Cellobionolactone was incubated with either FPLC-purified β-glucosidase (0.45 U) or dialyzed extracellular protein (1 mg) containing 0.45 U of β-glucosidase activity in acetate (50 mM, pH 5, 3 ml) for 60 min at 22°C. Aliquots (0.5 ml) were withdrawn periodically, and the amount of glucose released was determined as described above.

Comparison of cellobiose, cellobionolactone, and calcium cellobionate hydrolyses. Each substrate (7.5 mM) was incubated individually with the cellulose-binding form of β-glucosidase (0.5 U) in acetate buffer (50 mM, pH 5, 3 ml) for 15 min at 22°C, and the amount of glucose released was determined periodically.

Kinetics. Apparent K_m and k_{cat} values were calculated from double reciprocal plots. K_i values for glucose, cellobiose, gluconolactone, cellobionolactone, and cellobionate were determined with PNPG as the substrate. All kinetic experiments were performed at 22°C.

Papain hydrolysis of β-glucosidase. A homogeneous cellulose-binding form of β-glucosidase (0.58 mg) was incubated with papain (12.5 μg) in phosphate buffer (0.5 ml, 0.1 M, pH 7) containing 2 mM EDTA and 2 mM dithiothreitol at room temperature for 20 h. β-Glucosidase activity remaining at the end of this reaction was assayed with PNPG. The protein mixture was loaded on a microcrystalline cellulose column (3 by 0.5 cm) equilibrated with phosphate buffer (pH 7, 50 mM). The column was eluted first with phosphate buffer (4 ml) and then with water (4 ml). Fractions (0.75 ml) were collected and assayed for β-glucosidase activity. Phosphate-buffer-eluted proteins were further purified by FPLC using a Mono Q HR 5/5 column and 10 mM Tris-HCl, pH 8, with 1 M NaCl as the eluent. FPLC-purified protein was then analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Table 1 shows the results of the β-glucosidase purification procedure. SDS-PAGE analysis of FPLC-purified β-glucosidase indicated the presence of three bands corresponding to molecular weights of 96,000, 98,000, and 114,000 (Fig. 1). The highest-molecular-weight band appeared to be the major constituent. On further affinity fractionation with a microcrystalline cellulose column, 82% of the β-glucosidase activity bound to cellulose. SDS-PAGE analysis of the unbound proteins indicated the presence of only two bands with molecular weights of 96,000 and 98,000 (Fig. 1). The cellulose-bound β-glucosidase was quantitatively recovered from the column by elution with water, and SDS-PAGE analysis of this fraction indicated the presence of a single protein with a molecular weight of

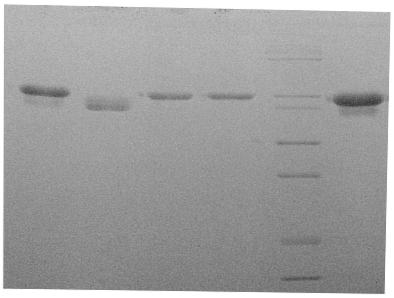

FIG. 1. SDS-PAGE of purified β-glucosidase from *P. chrysosporium*. Lanes 1 and 6, FPLC-purified β-glucosidase (3 and 5 μg); lane 2, non-cellulose-binding form of β-glucosidase (3 μg); lanes 3 and 4, cellulose-binding form of β-glucosidase (1.5 and 2 μg); lane 5, molecular weight markers (myosin, 200,000; β-galactosidase, 116,300; phosphorylase *b*, 97,400; bovine serum albumin, 66,300; glutamate dehydrogenase, 55,400; lactate dehydrogenase, 36,500; and carbonic anhydrase, 31,000).

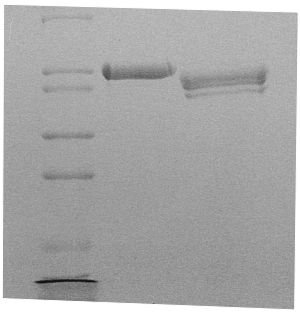

FIG. 3. SDS-PAGE of papain-treated β-glucosidase. Lane 1, molecular weight markers (myosin, 200,000; β-galactosidase, 116,300; phosphorylase *b*, 97,400; bovine serum albumin, 66,300; glutamate dehydrogenase, 55,400; lactate dehydrogenase, 36,500; and carbonic anhydrase, 31,000); lane 2, cellulose-binding β-glucosidase; lane 3, papain-treated cellulose-binding β-glucosidase.

114,000 (Fig. 1). Phosphate buffer (50 mM, pH 4.5), or phosphate buffer containing 10% cellobiose, 1% Nonidet P-40, 1 M NaCl, or 1 M urea, did not elute the enzyme from the column. The specific activity of β-glucosidase did not substantially increase after cellulose affinity chromatography. This procedure yielded a 130-fold-purified enzyme with a specific activity of 33 U/mg (Table 1). Purified β-glucosidase did not exhibit α-glucosidase or β-galactosidase activity, and it was devoid of cellobiose-oxidizing enzymes, cellobiose dehydrogenase, and cellobiose:quinone oxidoreductase (2, 5, 29). The neutral carbohydrate content of the cellulose-binding β-glucosidase was 18.5%. Smith and Gold (24) partially purified an extracellular β-glucosidase with a molecular weight of 90,000 from *P. chrysosporium*. Deshpande et al. (10) purified a number of extracellular β-glucosidases from *P. chrysosporium* (*S. pulverulentum*), and none of them correspond to the β-glucosidases reported in the present study. Earlier investigations did not examine the cellulose binding characteristics of β-glucosidase (10, 24).

Cellulose-bound β-glucosidase and the free enzyme hydrolyzed PNPG at the same rate (Fig. 2). This activity was not due to the release of glucosidase from cellulose, because when the cellulose suspension was centrifuged and the supernatant was replaced with fresh buffer and PNPG, the observed rate of PNPG hydrolysis was equivalent to that of the first cycle. This result suggests that the catalytic site of β-glucosidase is not involved in cellulose binding. When the cellulose-binding β-glucosidase was treated with papain, there was no loss of activity. However, only 26% of the papain-treated enzyme was bound to cellulose. In contrast, 90% of the β-glucosidase from a control reaction mixture, which did not receive papain, was bound to cellulose. Papain-treated β-glucosidase was purified with a cellulose column to separate the nonbinding form from the binding form and by FPLC to separate the glucosidase from papain. Fractions containing glucosidase activity were combined and analyzed by SDS-PAGE. Three bands with molecular weights in the range of 95,000 to 97,000 were observed (Fig. 3). Thus, our proteolysis experiment suggests that the lower-molecular-weight non-cellulose-binding forms of *P. chrysosporium* β-glucosidase are most probably formed from the higher-molecular-weight cellulose-binding form via extracellular proteolytic hydrolysis. Cellulose-degrading cultures of *P. chrysosporium* are known to produce extracellular proteases (3, 12). The cellulose binding characteristic is generally observed only with cellulases. These enzymes are organized into two domains, a cellulose-binding domain and a catalytic domain (14). The cellulose-binding domain is usually located at the amino or carboxy terminus of the enzyme and is separated from the catalytic domain by a linker region enriched with proline, threonine, and serine (14). β-Glucosidase of *P. chrysosporium* also might have a similar two-domain organization consisting of a cellulose-binding domain and a catalytic domain. This proposal is supported by the observation that papain hydrolysis of β-glucosidase generates an enzyme moiety which retains all of the original activity but has lost the ability to bind to cellulose.

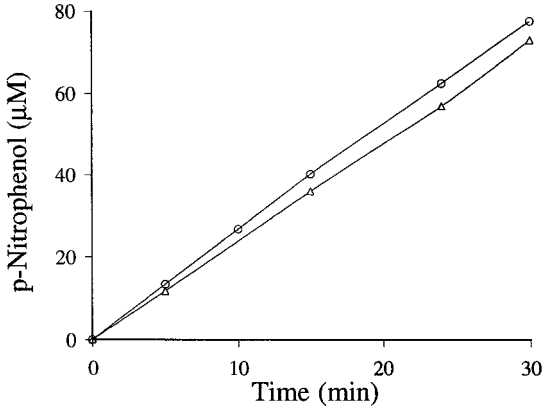

FIG. 2. Comparison of PNPG hydrolyses by free (○) and microcrystalline cellulose-bound (△) β-glucosidase. β-Glucosidase (0.06 U) was incubated with 1 mM PNPG in 50 mM phosphate, pH 4.5. *p*-Nitrophenol release was determined as described in Materials and Methods.

Cellulose-binding β-glucosidase exhibited maximum PNPG hydrolysis activity at pH 4 to 5.2. It was very stable at pH 4 to 8 and at temperatures below 45°C. The K_ms for PNPG and cellobiose hydrolyses were 0.096 and 2.3 mM, respectively. The calculated k_{cat} values for PNPG and cellobiose hydrolyses were 132 and 50 s^{-1}, respectively. PNPG and cellobiose hydrolyses were competitively inhibited by glucose (K_i = 0.27 mM), gluconolactone (K_i = 0.004 mM), calcium cellobionate (K_i = 0.4 mM), and cellobionolactone (K_i = 0.03 mM). A comparison of K_m values for PNPG and cellobiose for the β-glucosidases from several cellulolytic fungi indicates that *P. chrysosporium* β-glucosidase exhibits the lowest K_m for PNPG and an appar-

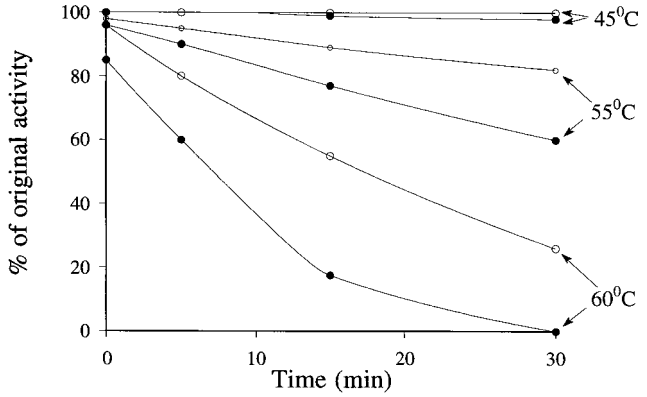

FIG. 4. Temperature stability of cellulose-binding (○) and -nonbinding (●) β-glucosidase from *P. chrysosporium*. β-Glucosidase (0.24 U) was incubated at a specified temperature in 50 mM phosphate, pH 4.5, and the enzyme activity was monitored at regular intervals.

ently high K_m for cellobiose (6, 7, 13, 17, 20, 22, 23, 26, 30, 31). A similar comparison of K_i values for glucose and gluconolactone suggests that *P. chrysosporium* glucosidase exhibits very low K_is for these inhibitors (6, 7, 13, 17, 20, 22, 23, 26, 30, 31). The kinetic properties of non-cellulose-binding forms were similar to those of the binding form (data not shown). However, the cellulose-binding form appears to be more thermostable than the non-cellulose-binding forms (Fig. 4).

Hydrolysis of cellobiose, cellobionolactone, and calcium cellobionate was determined by monitoring glucose production, and the observed initial velocities were 8.4, 0.36, and 0.6 μmol min^{-1} mg^{-1}, respectively (Fig. 5). The amount of glucose

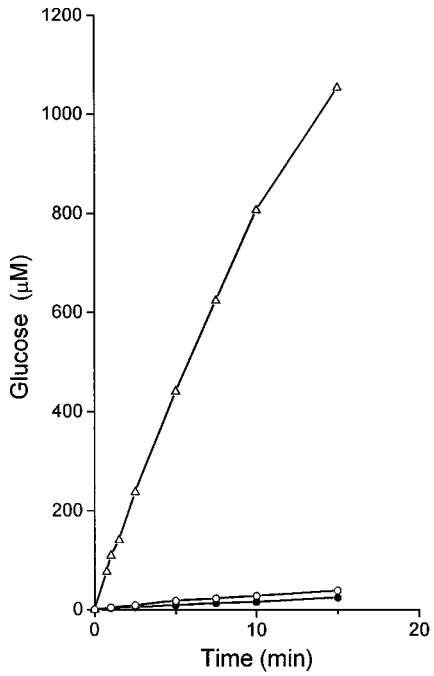

FIG. 5. Comparison of cellobiose (△), cellobionolactone (●), and calcium cellobionate (○) hydrolyses by β-glucosidase from *P. chrysosporium*. β-Glucosidase (0.5 U) was incubated with each substrate (7.5 mM) individually in 50 mM acetate, pH 5. Production of glucose was determined by the glucose oxidase-peroxidase method.

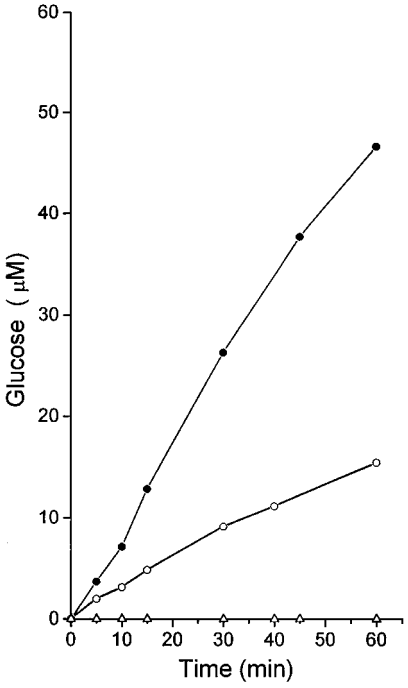

FIG. 6. Cellobionolactone hydrolysis by purified β-glucosidase and extracellular protein from cellulolytic cultures. FPLC-purified β-glucosidase (0.45 U) (○) or β-glucosidase (0.45 U) containing extracellular concentrate of a cellulolytic culture of *P. chrysosporium* (●) was incubated with cellobionolactone (2.5 mM) in 50 mM phosphate, pH 4.5. Glucose production was monitored by the glucose oxidase-peroxidase method (18). Levels of nonenzymatic hydrolysis (△) of cellobionolactone were also determined.

released by cellobiose hydrolysis was divided by two because two glucose units are generated for each unit of cellobiose hydrolyzed. Hydrolyses of cellobionolactone by homogeneous β-glucosidase and by dialyzed total extracellular proteins containing the same level of β-glucosidase activity were compared. The extracellular proteins hydrolyzed cellobionolactone about three times faster than the purified β-glucosidase (Fig. 6). It is possible that *P. chrysosporium* cellulases are capable of hydrolyzing cellobionolactone and that the increased cellobionolactone hydrolysis observed in our experiments with extracellular proteins was due to cellulases.

In summary, an extracellular cellulose-binding β-glucosidase from cellulose-degrading cultures of *P. chrysosporium* has been purified and characterized. Earlier, we demonstrated that cellobiose-oxidizing enzymes from *P. chrysosporium* bind tightly to microcrystalline cellulose (21). The exocellobiohydrolase of *P. chrysosporium* has been demonstrated to possess a cellulose-binding domain (9). Thus, the *P. chrysosporium* cellulolytic system is novel in that all of its known components bind to cellulose.

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