Production, Purification, and Characterization of a Highly Glucose-Tolerant Novel β-Glucosidase from *Candida peltata*

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Received 1 March 1996/Accepted 15 June 1996

*Candida peltata* (NRRL Y-6888) produced β-glucosidase when grown in liquid culture on various substrates (glucose, xylose, l-arabinose, cellobiose, sucrose, and maltose). An extracellular β-glucosidase was purified 1,800-fold to homogeneity from the culture supernatant of the yeast grown on glucose by salting out with ammonium sulfate, ion-exchange chromatography with DEAE Bio-Gel A agarose, Bio-Gel A-0.5m gel filtration, and cellobiose-Sepharose affinity chromatography. The enzyme was a monomeric protein with an apparent molecular weight of 43,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration. It was optimally active at pH 5.0 and 50°C and had a specific activity of 108 μmol · min⁻¹ · mg of protein⁻¹ against p-nitrophenyl-β-D-glucoside (pNPPG). The purified β-glucosidase readily hydrolyzed pNPPG, cellobiose, celletriose, cellettetraose, cellopentaose, and cellohexaose, with *K₅₀* values of 2.3, 66, 39, 35, 21, and 18 mM, respectively. The enzyme was highly tolerant to glucose inhibition, with a *K₅₀* of 1.4 M (252 mg/ml). Substrate inhibition was not observed with 40 mM pNPPG or 15% cellobiose. The enzyme did not require divalent cations for activity, and its activity was not affected by p-chloromercuribenzoate (0.2 mM), EDTA (10 mM), or diithiothreitol (10 mM). Ethanol at an optimal concentration (0.75%, vol/vol) stimulated the initial enzyme activity by only 11%. Cellobiose (10%, wt/vol) was almost completely hydrolyzed to glucose by the purified β-glucosidase (1.5 U/ml) in both the absence and presence of glucose (6%). Glucose production was enhanced by 8.3% when microcrystalline cellulose (2%, wt/vol) was treated for 24 h with a commercial cellulase preparation (cellulase, 5 U/ml; β-glucosidase, 0.45 U/ml) that was supplemented with purified β-glucosidase (0.4 U/ml).

More than one billion gallons (ca. 4 × 10⁹ liters) of ethanol are produced annually in the United States, with approximately 95% derived from fermentation of cornstarch (6). Various cellulosic agricultural residues such as corn stover, straw, and bagasse can also serve as low-value and abundant feedstocks for production of fuel alcohol. Currently, the utilization of cellulosic biomass to produce fuel ethanol presents significant technical and economic challenges, and its success depends largely on the development of highly efficient and cost-effective biocatalysts for conversion of pretreated biomass to fermentable sugars.

The enzymatic saccharification of cellulosic material to glucose involves the synergetic action of at least three different enzymes: (i) endo-β-1,4-glucanase (EC 3.2.1.4), (ii) exo-cellobiohydrolase (EC 3.2.1.91), and (iii) β-glucosidase (β-D-glucosidase glucohydrolase; EC 3.2.1.21). Endoglucanase and exo-cellobiohydrolase act synergistically upon cellulose to produce cellobiose, which is then cleaved by β-glucosidase to glucose. Both endoglucanase and cellobiohydrolase activities are often inhibited by cellobiose (9, 15, 39). β-Glucosidase reduces cellobiose inhibition by hydrolyzing cellobiose to glucose, which allows the celluloytic enzymes to function more efficiently (31, 41). However, most microbial β-glucosidases that catalyze the hydrolysis of cellobiose are very sensitive to glucose inhibition, which limits their activity (13, 32). Furthermore, the enzyme is also inhibited by its own substrate, cellobiose (33, 39). In this respect, the availability of β-glucosidase insensitive to glucose and cellobiose inhibition will have a significant impact on the enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol.

Recently, we have screened 48 yeast strains belonging to the genera *Candida*, *Debaryomyces*, *Kluyveromyces*, and *Pichia* (obtained from the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, Ill.) for production of extracellular glucose-tolerant and thermophilic β-glucosidases (30). Enzymes from 15 yeast strains showed very high glucose tolerance (<50% inhibition in 30% [wt/vol] glucose). In this paper, we report on the production, purification, and properties of a highly glucose-tolerant novel β-glucosidase from *Candida peltata*.

**MATERIALS AND METHODS**

*Methanol.* All saccharides, all aryl-glycosides, salicin, molecular weight markers for gel filtration, and the glucose detection kit were obtained from Sigma Chemical Co., St. Louis, Mo. Molecular weight markers and precast gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), DEAE Bio-Gel A agarose, Bio Gel A-0.5m gel, and an Aminex HPX 87C column for high-pressure liquid chromatography (HPLC) were purchased from Bio-Rad Laboratories, Hercules, Calif. Epoxy-activated Sepharose 6B was from Pharmacia LKB Biotechnology, Piscatway, N.J. Cellooligosaccharides (celletriose to cellohexaose) prepared by the method of Freer and Detroy (12) were kindly supplied by R. B. Hespell. Corn bran (Diestiller NU 20085) was obtained from Lauhoff Grain Company, Danville, III. Cytolase 121 was supplied by Genencor International, Rochester, N.Y. Yeast extract was purchased from Difco Laboratories, Detroit, Mich. All other chemicals used were analytical grade.

*Yeast strain, medium, and culture conditions.* The yeast strain (*C. peltata* NRRL Y-6888) used in this study was obtained from the ARS Culture Collection. The medium (34) used for production of β-glucosidase had the following composition (per liter): 10 ml of solution A, 10 ml of solution B, 100 ml of solution C, 10 g of yeast extract, and 10 g of glucose. Solution A was a trace mineral solution having the following ingredients (per liter): 1.1 g of CaO, 0.4 g of ZnO, 0.5 g of FeCl₃ · 6H₂O, 0.25 g of CuSO₄ · 5H₂O, 0.24 g of CoCl₂ · 6H₂O, 0.14 g of MnCl₂ · 4H₂O, 0.12 g of MgSO₄ · 7H₂O, 0.02 g of Na₂MoO₄ · 2H₂O, and 0.02 g of KI.
0.06 g of \( \text{H}_2\text{BO}_3 \), and 13 ml of concentrated HCl. Solution B (per liter) was composed of 10.1 g of MgO and 45 ml of concentrated HCl. Solution C (per liter) contained 64 g of urea, 12 g of \( \text{KH}_2\text{PO}_4 \), and 1.5 g of NaCl. Substrates were sterilized separately. The pH was adjusted to 5.0 with 1 M HCl before concentration. A 250-ml Erlenmeyer flask containing 100 ml of medium with glucose (1%) was inoculated with a loopful of cells taken from a stock slant and incubated at 28°C on a rotary shaker (200 rpm) for 3 days. The shake flasks (250-ml Erlenmeyer flask containing 100 ml of medium) were inoculated with 2 ml of this culture and cultivated on a rotary shaker (200 rpm) at 28°C. After 4 days, the cells were removed from the culture broth by centrifugation (18,000 \( \times \) g, 20 min). The resulting supernatant solution was used as the crude enzyme preparation.

Results of the purification procedures of an extracellular \( \beta \)-glucosidase from the culture supernatant of \( C. \) peltata grown on glucose. The enzyme was purified, 1,800-fold to homogeneity with an overall enzyme yield of 36% and a specific activity of 108 U mg of protein \(^{-1}\). Only one form of \( \beta \)-glucosidase was detected during the purification steps. No change in cellobiose-hydrolyzing activity was observed among the pNP\( \beta \)-glucosidase.

### Table 1. Growth and \( \beta \)-glucosidase production by \( C. \) peltata Y-6888 on various substrates

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth (( A_{660} ))</th>
<th>( \beta )-Glucosidase produced (mU/ml of culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole broth</td>
<td>4.2</td>
<td>18</td>
</tr>
<tr>
<td>Supernatant</td>
<td>14.2</td>
<td>16</td>
</tr>
</tbody>
</table>

* Cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of medium at 28°C for 4 days. The initial pH of the medium was 5.0 before inoculation.

### Table 2. Purification of \( \beta \)-glucosidase from \( C. \) peltata Y-6888

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg protein)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>7.094</td>
<td>392</td>
<td>0.06</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>2.215</td>
<td>365</td>
<td>0.16</td>
<td>93</td>
<td>2.7</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>175</td>
<td>286</td>
<td>1.6</td>
<td>73</td>
<td>27</td>
</tr>
<tr>
<td>DEAE Bio-Gel A agarose</td>
<td>7.8</td>
<td>231</td>
<td>29.6</td>
<td>59</td>
<td>494</td>
</tr>
<tr>
<td>Bio-Gel A 0.5m</td>
<td>2.3</td>
<td>163</td>
<td>70.9</td>
<td>42</td>
<td>1,182</td>
</tr>
<tr>
<td>Cellobiose-Sepharose</td>
<td>1.3</td>
<td>141</td>
<td>108</td>
<td>36</td>
<td>1,800</td>
</tr>
</tbody>
</table>
enzymes at each purification step in comparison with that of the crude enzyme. SDS-PAGE analysis of the purified enzyme indicated the presence of a single band when stained with Coomassie brilliant blue (Fig. 1).

**Characterization of β-glucosidase.**

(i) **Molecular weight.**

The molecular weight of the native β-glucosidase estimated by gel filtration on a Bio-Gel A-0.5m column was 43,500, and by SDS-PAGE analysis it was about 43,000 (Fig. 1), suggesting that the β-glucosidase was a monomeric protein.

(ii) **pH and temperature dependence.**

The pH stability and activity curves for β-glucosidase are shown in Fig. 2. The enzyme was fairly stable at pH 4.0 to 6.0 (30 min at 40°C), with 62% activity at pH 3.0 and 21% activity remaining at pH 7.0. It exhibited an optimal activity at pH 5.0, with 45% and 57% activities at pH 4.0 and 6.0, respectively. The effects of temperature on the stability and activity of the purified β-glucosidase are shown in Fig. 3. The enzyme in 50 mM acetate buffer (pH 5.0; 0.03 U/ml or 0.28 µg/ml) was fairly stable at temperatures up to 45°C for 30 min. It was inactivated upon incubation at 70°C for 30 min. The β-glucosidase (1.2 mg/ml) retained its full activity at 4°C after storage in 50 mM acetate buffer (pH 5.0) for more than 4 months. The enzyme displayed maximal activity at 50°C.

(iii) **Substrate specificity and kinetic analysis.**

Relative rates of hydrolysis of various substrates by the purified β-glucosidase were studied. The enzyme could hydrolyze pNPβG and cellobiose effectively. Salicin was hydrolyzed at 34% of that of cellobiose. The purified enzyme had very little (≤5%) or no activity on lactose, maltose, sucrose, and trehalose. It also had no or very little activity on p-nitrophenyl-α-D-glucoside, p-nitrophenyl-α-D-xyloside, p-nitrophenyl-β-D-cellobioside, p-nitrophenyl-α-L-arabinofuranoside, and p-nitrophenyl-β-D-glucuronide (≤5%). p-Nitrophenyl-β-D-galactoside was hydrolyzed at 6% of that of pNPβG. The reaction kinetics of the purified enzyme were determined from Lineweaver-Burk plots with pNPβG and cellooligosaccharides (cellobiose to cellotriose as substrates under optimal conditions (pH 5.0 and 50°C). The enzyme had apparent $K_m$ values of 2.3, 66, 39, 35, 21, and 18 mM and $V_{max}$ values of 221, 75, 32, 16, 8, and 5

µmol · min$^{-1}$ · mg of protein$^{-1}$ for the hydrolysis of pNPβG, cellobiose, cellotriose, cellotetraose, cellopentaose, and cello-hexaose, respectively.

(iv) **Inhibition by glucose, cellobiose, and other sugars.**

The study of inhibition by glucose was performed with pNPβG as the substrate. Glucose acted as a competitive inhibitor of
pNPβG hydrolysis with an inhibition constant (K_i) of 1.4 M (252 mg/ml) obtained at the intersection of the lines on the Dixon plot analysis (Fig. 4). Galactose, mannose, arabinose, fructose, xylose (each at 56 mM), sucrose, and lactose (each at 29 mM) did not inhibit the β-glucosidase activity. Substrate inhibition was not observed at all with either 40 mM pNPβG or 15% cellobiose.

(v) Inhibitors and activators. The effect of selective inhibitors or activators on β-glucosidase activity was examined. The enzyme was not dependent on Ca^{2+}, Mg^{2+}, Mn^{2+} (each at 5 mM), or Ce^{4+} (0.5 mM) for activity. Enzyme activity was not affected by EDTA (10 mM), dithiothreitol (10 mM), or p-chloromercuribenzoic acid (0.2 mM). Ethanol at optimal concentration had very little stimulating effect on the initial β-glucosidase activity. Substrate inhibition was not observed at all with either 40 mM pNPβG or 15% cellobiose.

(vi) Cellobiose hydrolysis. Figure 5 shows the rates of cellobiose (10% [wt/vol]) hydrolysis performed by purified β-glucosidase (1.5 U/ml) in both the presence and absence of glucose (6% [wt/vol]). Both cellobiose degradation and glucose production were quantified by HPLC. Glucose was detected as the only reaction product from cellobiose during the course of the reaction. The enzyme was able to function very well in the presence of glucose and hydrolyzed about 90% cellobiose to glucose in 144 h.

(vii) Synergism with cellulase. The effect of purified β-glucosidase (0.4 U/ml) supplementation on microcrystalline cellulose (Sigmacell type 50; 2% [wt/vol]) hydrolysis at pH 5.0 and 50°C by a commercial cellulase preparation (CytoZyme 123, cellulase [5 U/ml], and β-glucosidase [0.45 U/ml]) was examined by comparing the nature and quantity of the soluble products formed in the absence and presence of the enzyme. The reaction products were analyzed by HPLC. After 24 h, there was an 8.3% increase in the production of glucose from Sigmacell by the cellulase preparation supplemented with the β-glucosidase. The β-glucosidase itself had no activity on microcrystalline cellulose. Thus, the β-glucosidase from C. peltata had a synergistic interaction with cellulase to increase the efficiency of glucose production from cellulose.

**DISCUSSION**

To our knowledge, these findings represent the first report on the production, purification, and characterization of a yeast β-glucosidase having such a high tolerance to glucose (K_i, 1.4 M). The level of production of β-glucosidase by C. peltata NRRL Y-6888 depended on the carbohydrate source (Table 1). It is interesting that the highest enzyme production (117 mU/ml) was observed in xylose-grown culture broths. Provision of cellobiose as the carbohydrate source was not required at all for enzyme production. The yeast produced β-glucosidase when grown on all carbon sources examined. Thus, the β-glucosidase of C. peltata seemed to be a constitutive enzyme, regardless of the presence or absence of cellobiose. The yeast produced β-glucosidase significantly even when grown on 20% glucose. It also produced ethanol from glucose. The decrease in enzyme activity in the culture broth when the yeast was grown at a higher glucose concentration may be due to the alcohol produced by the yeast, which inhibited the enzyme activity when assayed with the culture broth. This indicates that the enzyme synthesis might not be repressed by glucose.

The majority of the β-glucosidase activity was extracellular. An extracellular β-glucosidase was purified 1,800-fold from the glucose-grown cell-free culture broth by a four-step procedure involving ammonium sulfate treatment, DEAE Bio-Gel A agarose ion-exchange chromatography, Bio-Gel A-0.5m gel filtration, and affinity chromatography on cellobiose-Sepharose 6B (Table 2). The enzyme was tightly adsorbed onto the affinity matrix and was eluted only by changing the buffer, changing the pH, and adding a strong salt (2 M NaCl) concentration. Only one active form of enzyme was detected during the purification procedures. Multiple forms of β-glucosidase have been found in the culture broth of a variety of microbes (7, 13, 19, 22, 24, 03). The specific activity of β-glucosidase from C. peltata was 108 U/mg of protein under optimal conditions (pH 5.0 and 50°C). The specific activities of purified β-glucosidases from other microorganisms range from 5 to 979 U/mg of protein (1, 11, 17, 19, 37).

The molecular weight (43,000) (Fig. 1) of the β-glucosidase...
from *C. peltata* is similar to that of the extracellular β-glucosidases from *Clostridium thermocellum* (*M*, 43,000), a *Monilia* sp. (*M*, 46,600), a *Pironymae* sp. (*M*, 45,000), *Trichoderma koningii* (*M*, 39,800), and intracellular β-glucosidase from *Sporotrichum thermophile* (*M*, 40,000) (1, 3, 4, 35, 38). Molecular weights of β-glucosidases from various microbial sources vary from 39,800 to 480,000 (10, 32, 35, 38). The optimal activity of the purified enzyme was observed at pH 5.0 and 50°C (Fig. 2 and 3). The optimal pHs and temperatures of β-glucosidases from various microbial sources range between 3 and 7 and 40 and 105°C, respectively (9, 16, 32, 39).

Glycosidases may be divided into three groups on the basis of substrate specificity: (i) aryl-β-glucosidase (which hydrolyzes exclusively aryl-β-glucosides), (ii) celllobiose (which hydrolyzes cellobiose and short-chain cellodextrins only), and (iii) broad-spectrum β-glucosidases (which show activity on both substrate types). The last type is the most commonly observed group in cellulytic microorganisms (7, 13, 25, 32, 33, 39). The β-glucosidase from *C. peltata* is a broad-specificity type since it hydrolyzed cellobiose, cellooligosaccharides, and pNPβG. The enzyme had very little β-xylanosidase, 1-arabinofuranosidase, and β-glucuronidase activity (less than 5% of that of β-glucosidase). It hydrolyzed p-nitrophenyl-β-d-galactoside at 6% of that of pNPβG. Although there are a few exceptions (1, 11, 24, 36, 40), the competitive inhibition of β-glucosidase by glucose is a quite common phenomenon (13, 26, 32, 39). Most microbial enzymes show inhibition constants (K) of 0.6 to 8 mM for glucose (24). The β-glucosidases from *Sporotrichum thermophile*, a *Monilia* sp., *Fusarium oxysporum*, *Neocallimastix frontalis*, *Botrytis cinerea*, and *Streptomyces* sp. strain QM-BS14 were competitively inhibited by glucose, with Ks of 0.5, 0.67, 2.05, 5.5, 10.5, 65 mM, respectively (4, 8, 10, 13, 19, 28). Figure 4 shows that glucose caused competitive inhibition of the β-glucosidase of *C. peltata*, with a K of 1.4 M (252 mg/ml), which indicates that the enzyme is highly tolerant to glucose. Glucose inhibited the β-glucosidase-catalyzed reaction of *Trichoderma viride* cellulase in a mixed inhibition pattern with a competitive character (23). The inhibition of β-glucosidase from *Pyrococcus furiosus* by glucose was almost negligible, with a K of 300 mM (16). Aryl-β-glucosidase of *Trichoderma* spp. was totally inhibited by 1% glucose, and *Microbispora bispora* aryl-β-glucosidase was 35, 66, and 79% inhibited by 10, 20, and 30% glucose, respectively (36). A cloned β-glucosidase (BβG) from *Microbispora bispora* was also activated two- to threefold in the presence of 2 to 5% (0.1 to 0.3 M) glucose and did not become inhibited until the glucose concentration reached about 40% (40). One β-glucosidase from *Streptomyces* sp. was activated twofold by 1.8% glucose, and another one from the same strain was 50% inhibited by 18% glucose (24). Recently, Perez-Pons et al. (29) reported that glucose in the range of 0.45 to 3.6% (25 to 200 mM) enhanced the rate of pNPβG hydrolysis by a β-glucosidase isolated from a *Streptomyces* sp.

None of the divergent cations tested had significant stimulatory or inhibitory effects on β-glucosidase activity. Strong inhibition of β-glucosidases is generally observed with p-chloromercuribenzoate, a thiol-specific inhibitor (1, 29). However, the β-glucosidase activity from *C. peltata* was unaffected by p-chloromercuribenzoate. This suggests that sulfhydryl groups are not involved in the catalytic center of the enzyme. The β-glucosidases from *Pyrococcus furiosus* and *Aureobasidium pullulans* are also unaffected by thiol-specific inhibitors (16, 31). Substrate inhibition by cellobiose is a common property of β-glucosidase from *Trichoderma* spp. and other microorganisms (7, 28, 32, 33, 35). Celllobiose strongly inhibited its own hydrolysis by β-glucosidase from a *Pyromyces* sp. at concentra-