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Purification and Biochemical Properties of a Glucose-Stimulated β-D-Glucosidase Produced by *Humicola grisea* var. *thermoidea* Grown on Sugarcane Bagasse

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The effect of several carbon sources on the production of mycelial-bound β-glucosidase by *Humicola grisea* var. *thermoidea* in submerged fermentation was investigated. Maximum production occurred when cellulose was present in the culture medium, but higher specific activities were achieved with cellobiose or sugarcane bagasse. Xylose or glucose (1%) in the reaction medium stimulated β-glucosidase activity by about 2-fold in crude extracts from mycelia grown in sugarcane bagasse. The enzyme was purified by ammonium sulfate precipitation, followed by Sephadex G-200 and DEAE-cellulose chromatography, showing a single band in PAGE and SDS-PAGE. The β-glucosidase had a carbohydrate content of 43% and showed apparent molecular masses of 57 and 60 kDa, as estimated by SDS-PAGE and gel filtration, respectively. The optimal pH and temperature were 6.0 and 50°C, respectively. The purified enzyme was thermostable up to 60 min in water at 55°C and showed half-lives of 7 and 14 min when incubated in the absence or presence of 50 mM glucose, respectively, at 60°C. The enzyme hydrolyzed *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, lactose, and cellobiose. The best synthetic and natural substrates were *p*-nitrophenyl-β-D-fucopyranoside and cellobiose, respectively. Purified enzyme activity was stimulated up to 2-fold by glucose or xylose at concentrations from 25 to 200 mM. The addition of purified or crude β-glucosidase to a reaction medium containing *Trichoderma reesei* cellulases increased the saccharification of sugarcane bagasse by about 50%. These findings suggest that *H. grisea* var. *thermoidea* β-glucosidase has a potential for biotechnological applications in the bioconversion of lignocellulosic materials.

***Keywords*:** cellobiase, glucose-stimulated β-D-glucosidase, *H. grisea*, sugarcane bagasse, agricultural residues, thermophilic fungi

Cellulose is the most abundant renewable natural biological resource, and the production of biobased products and bioenergy from less costly lignocellulosic materials is today the focus of great interest by numerous researchers in several countries (Bhat and Bhat, 1997; Lynd *et al.*, 2002; Zhang *et al.*, 2006; Kumar *et al.*, 2008).

Chemically, cellulose consists of a homopolysaccharide of β-D-glucopyranose residues linked by β-(1→4)-glycosidic bonds, and cellobiose is its smallest repetitive unit (Lynd *et al.*, 2002; Kumar *et al.*, 2008). The enzymatic hydrolysis of cellulose is carried out by the concerted and synergistic action of cellulases, which may be divided into three groups: endo-1,4-β-glucanases (EC 3.2.1.4), exo-1,4-β-glucanases or cellobiohydrolases (EC 3.2.1.91), and 1,4-β-glucosidases (EC 3.2.1.21). Whereas endoglucanases randomly attack the cellulose chain at internal bonds, exoglucanases catalyze the release of cellobiose from its reducing and nonreducing ends. Cellobiose and short cellooligosaccharides are then converted to glucose monomers by the action of β-glucosidases (Beguin and Aubert, 1994; Kumar *et al.*, 2008). As a rule, endo- and exoglucanases are strongly inhibited by cellobiose, and thus β-glucosidases are considered the rate-limiting factors of most cellulolytic systems (Bhatia *et al.*, 2002; Lynd *et al.*, 2002).

Most β-glucosidases are also strongly inhibited by glucose, thus restricting the complete saccharification of cellulose (Bhatia *et al.*, 2002; Lynd *et al.*, 2002; Karnchanatat *et al.*, 2007; Kaur *et al*., 2007; Bhiri *et al.*, 2008; Yang *et al.*, 2008; Yoon *et al.*, 2008). However, in the last years, a few microbial β-glucosidases tolerant to (Riou *et al.*, 1998; Yang *et al.*, 2008) or stimulated by (Zanoelo *et al*., 2004; Sonia *et al*., 2008) glucose have been identified. Apart from product inhibition, filamentous thermophilic fungi are recognized as efficient producers of β-glucosidases with high thermal stability, a very interesting property for industrial purposes (Peralta *et al*., 1997; Venturi *et al*., 2002; Yang *et al.*, 2008; Yoon *et al*., 2008).

The major bottlenecks for industrial application of cellulases

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and β-glucosidases are their hydrolytic efficiency and the high cost of enzyme production (Gusakov *et al*., 2007). Substantial cost reduction can result from choosing microorganisms with high rates of enzyme production, which can be grown on cheap and easily available substrates. Indeed, several studies indicate that the carbon source is one of the most important factors affecting the production cost and yield of cellulase (Gao *et al*., 2008). In this context, lignocellulosic biomass, in particular, agroindustrial residues, such as sugarcane bagasse, corn cob, wheat bran, and rice straw, are now increasingly being investigated as carbon sources for microbial enzyme production, especially cellulases (El-Hawary *et al.*, 2001; El-Hawary and Mostafa, 2001; Kaur *et al.*, 2006; Zhang *et al.*, 2006; Gao *et al*., 2008; Leite *et al*., 2008; Sonia *et al*., 2008; Yang *et al.*, 2008).

The present study describes the purification and some biochemical properties of a glucose- and xylose-stimulated β-D-glucosidase produced by the thermophilic fungus *H. grisea* var. *thermoidea*. When grown in a liquid medium supplemented with sugarcane bagasse as the sole carbon source, this mold is a good producer of this enzyme, which also showed good thermal stability and elevated catalytic efficiency for cellobiose.

Materials and Methods

Organism and growth conditions

The *Humicola* strain was isolated from Brazilian soil and classified as *H. grisea* var. *thermoidea* on the basis of its morphological and physiological characteristics, according to Cooney and Emerson (1964). The fungus was maintained in the laboratory at 40°C, on slants of solid medium containing 4% oatmeal baby food (Quaker, Brazil).

Cellular distribution of glucose-stimulated β-D-glucosidase activity

Subcellular localization of the enzyme was evaluated according to the procedure of Zanoelo *et al*. (2004). Briefly, the fraction of total enzyme bound to the hyphal surface was estimated by assaying the activity of a sample of intact mycelium rinsed with chilled water (total volume equivalent to that of the culture medium). The fraction of enzyme loosely bound to the mycelium was estimated after extensively washing the mycelium with 20 equivalent culture volumes of chilled water. After destroying the tightly bound enzyme by treatment of the intact, extensively washed mycelium with 0.1 M HCl at 0°C (Mandels, 1953), the intracellular enzyme fraction was estimated in the supernatant fraction obtained after grinding with glass beads, as described below. The activity in each fraction was assayed in the presence of 100 mM glucose to entirely inactivate other β-glucosidases produced by *H. grisea* that are glucose-sensitive (Peralta *et al*., 1997).

Enzyme induction

Conidia collected from 10-day-old cultures were inoculated into a liquid medium of the following composition, adjusted to pH 6.0 with HCl: 0.1% $CaCO_3$, 0.1% peptone, 0.25% gelatin, 0.8% yeast extract, 0.5% NaCl, and 1% of the desired carbon source. The cultures were incubated at 40°C in a rotary shaker at 145 rpm for an adequate time interval.

Preparation of crude enzyme

After growth, mycelia were harvested by filtration, washed, blotted using filter paper, and stored at -20°C for at least 2 h. Mycelial pads were then ground in a porcelain mortar with glass beads at 4°C, and proteins were extracted from the disrupted cells with chilled deionized water (15 ml/g mycelium). The slurry was centrifuged at 8,000×g for 20 min at 4°C, and the supernatant was used as the crude enzyme preparation.

Enzyme assays

β-D-Glucosidase activity was routinely assayed at 50°C in 50 mM sodium phosphate buffer, pH 6.0, using 5 mM *p*-nitrophenyl-β-D-glucopyranoside (pNP-Glc) as substrate. After adequate time intervals, the reaction was interrupted by the addition of two volumes of saturated sodium tetraborate solution, and the hydrolysis rates were estimated by quantifying the liberation of *p*-nitrophenolate ion ($\varepsilon_{410nm,\ pH\ 12}$=17,500 $M^{-1}cm^{-1}$). Enzymatic activity on other aryl-glucosides, such as *p*-nitrophenyl-β-D-galactopyranoside (pNP-Gal), *o*-nitrophenyl-β-D-galactopyranoside (oNP-Gal), *p*-nitrophenyl-β-D-xylopyranoside (pNP-Xyl), *p*-nitrophenyl-β-D-fucopyranoside (pNP-Fuc), *p*-nitrophenyl-α-D-galactopyranoside (pNP-α-Gal), and *p*-nitrophenyl-α-D-glucopyranoside (pNP-α-Glc), was estimated similarly under the same conditions.

Hydrolysis of cellobiose and lactose (10 mM) was also assayed in 50 mM sodium phosphate buffer, pH 6.0, at 50°C, and the glucose released was detected by the glucose oxidase method (Bergmeyer and Bernt, 1974) or by high-performance liquid chromatography (HPLC). Hydrolytic activity against the polysaccharides carboxymethylcellulose (CM-cellulose), microcrystalline cellulose (Avicel®, Fluka Chemical Co., Germany), oat-spell xylan, starch, and pectin (1%, w/v) was assayed under the same conditions, and the reducing sugars released were quantified using the dinitrosalicylic acid (DNS) method, as described by Miller (1959).

In all experiments, the enzyme was conveniently diluted, and the reaction time was adjusted to accomplish initial-velocity measurements. One enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol of product per min. Specific activity was defined as U/mg protein.

Enzymatic hydrolysis of sugarcane bagasse was carried out in hermetically closed 100-ml Erlenmeyer flasks at 50°C under orbital agitation (100 rpm). The reaction medium was 50 mM sodium acetate buffer, pH 5.0, containing 6 mg/ml sugarcane bagasse and 2 mM sodium azide in a final volume of 50 ml. The reaction was initiated by the addition of 10 filter paper units (FPU) of cellulase activity (crude extracellular extract) from *Trichoderma reesei* mutant RP-98 (cellulase hyperproducer strain) or a mixture containing 10 FPU cellulase activity from *T. reesei* and 0.1 U of purified *H. grisea* var. *thermoidea* β-glucosidase. Alternatively, the purified enzyme was substituted by an aliquot of a crude enzyme preparation containing 0.1 U β-glucosidase activity. Aliquots of 2.0 ml were withdrawn after desired time intervals, and reducing sugars were estimated by the DNS method.

Purification of β-D-glucosidase

H. grisea var. *thermoidea* was cultivated for 20 h at 40°C and 145 rpm in liquid medium (0.1% $CaCO_3$, 0.1% peptone, 0.25% gelatin, 0.8% yeast extract, and 0.5% NaCl) supplemented with 1% raw sugarcane bagasse as the carbon source. The bagasse was previously washed with deionized water until reducing sugars were not detectable using the DNS method and dried in a stove at 40°C before storage at room temperature. The crude enzyme preparation was adjusted to 75% saturation with solid ammonium sulfate under stirring. After incubating overnight at 4°C, the precipitate formed was collected by centrifugation (10,000×g, 30 min, 4°C), dissolved in a small volume of

deionized water, exhaustively dialyzed against deionized water, and lyophilized. The lyophilized material was dissolved in 5.0 ml of 50 mM potassium phosphate buffer, pH 6.0 (buffer A). After removal of undissolved material by centrifugation, the supernatant was applied to a Sephadex G-200 column (4.5×40.0 cm) equilibrated with buffer A and eluted with the same buffer at a flow rate of 2.5 ml/h. Fractions showing high β-D-glucosidase activity were pooled and applied to a DEAE-cellulose column (16×1.5 cm) equilibrated in buffer A and eluted at a flow rate of 18.0 ml/h with a linear gradient (0-200 mM) of NaCl in the same buffer. Pooled active fractions were dialyzed against deionized water, lyophilized, and stored at -20°C.

Polyacrylamide gel electrophoresis

Electrophoresis under nondenaturing conditions (PAGE) was carried out according to the method of Davis (1964) in 7% acrylamide slab gels. SDS-PAGE was carried out using 7.5% acrylamide gels, according to the procedure of Weber and Osborn (1969). Molecular weight markers used were bovine serum albumin (66,000 Da), ovalbumin (46,000 Da), carbonic anhydrase (29,000 Da), and trypsin inhibitor (20,000 Da). Protein bands were stained with Coomassie Blue. Visualization of β-D-glucosidase activity in PAGE gels was carried out using 6-bromo-2-napthyl-β-D-glucopyranoside as substrate, according to the description of Peralta *et al*. (1990).

Estimation of molecular mass

The apparent native molecular mass of the purified β-D-glucosidase was estimated by gel filtration using a Bio-Sil SEC-400 HPLC column (Bio-Rad, USA). A sample of the purified enzyme was injected onto the column (0.78×30 cm), equilibrated and eluted (25°C, 1.0 ml/min) with 100 mM sodium phosphate buffer, pH 6.8, containing 150 mM sodium chloride and 10 mM azide. Each fraction (1.0 ml) was assayed for β-D-glucosidase activity. The void volume was determined using Blue dextran, and the molecular mass markers used were γ-globulin (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.3 kDa).

Determination of neutral carbohydrates and protein

Total neutral carbohydrates were determined according to the procedure described by Dubois *et al*. (1956), using D-mannose as the standard. Protein concentrations were estimated by the method of Lowry *et al*. (1951), using bovine serum albumin as the standard.

Determination of kinetic parameters

The maximum velocities (V_M) and Michaelis-Menten constants (K_M) were calculated by a nonlinear regression method using the SigrafW software (Leone *et al*., 2005).

Results and Discussion

Influence of the carbon source on the production of β-D-glucosidase activity

The effects of various carbon sources on β-D-glucosidase activity production by *H. grisea* var. *thermoidea* were evaluated using pNP-Glc as substrate (Table 1). The highest levels of total activity were obtained in liquid media supplemented with Avicel (36.87±2.21 U) or CM-cellulose (30.03±1.80 U). Some agroindustrial residues, such as corncob (20.25±1.42 U) and sugarcane bagasse (12.00±1.08 U), were also relatively good activity inducers, in addition to wheat bran (24.27±2.18 U) and cellobiose (11.89±0.95 U). Elevated specific activities were obtained for both cellobiose (3.28±0.36 U/mg) and sugarcane bagasse (2.93±0.35 U/mg) when used as carbon sources. Glucose, sucrose, fructose, and starch were the worst carbon sources for enzyme production. Other carbon sources, such as lactose, glycerol, xylan, filter paper, rice straw, and orange peels, considerably increased enzyme production and specific activity when compared to glucose. Addition of 1% glucose to culture media containing Avicel, CM-cellulose, corncob, sugarcane bagasse, wheat bran, or cellobiose severely repressed the production of the enzyme, reaching levels close to that obtained with glucose alone. The presence of cycloheximide (80 μg/ml) inhibited the production of β-D-glucosidase, suggesting a requirement for *de novo* protein synthesis.

Table 1. Effects of several carbon sources on ß-D-glucosidase production by *H. grisea* var. *thermoidea*

Carbon source (1%)	Total units (U)	Specific activity (U/mg)
Glucose	0.82±0.12	0.07±0.01
Fructose	0.96±0.13	0.08±0.01
Sucrose	0.43±0.08	0.06±0.01
Lactose	2.60±0.21	0.37±0.03
Maltose	1.41±0.17	0.33±0.03
Cellobiose	11.89±0.95	3.28±0.36
Starch	0.37±0.06	0.08±0.01
Glycerol	2.09±0.17	0.39±0.03
Xylan	3.94±0.63	0.23±0.04
Filter paper	1.92±0.27	0.65±0.09
Rice straw	2.30±0.39	0.94±0.16
Orange peels	5.34±0.69	1.67±0.22
Avicel	36.87±2.21	1.82±0.11
Corncob	20.25±1.42	2.27±0.15
Sugarcane bagasse	12.00±1.08	2.93±0.35
Wheat bran	24.27±0.21	1.98±0.18
CM-cellulose	30.03±1.80	1.17±0.07

β-D-Glucosidase activity was estimated in 50 mM sodium phosphate buffer, pH 6.0, at 50°C, using 5 mM pNP-Glc as substrate. Data are the Mean±SD of three different cultures in liquid medium (25 ml) for 20 h at 40°C and 145 rpm (n=3).

The best culture conditions for β-D-glucosidase production by *H. grisea* var. *thermoidea* with sugarcane bagasse were 40°C under orbital agitation (145 rpm) for 20 h. Sugarcane bagasse is generated in massive quantities in Brazilian ethanol and sugar plants, where it is primarily burned for energy production. The excess may, however, represent a cheap carbon source for the production of enzymes of biotechnological interest, such as the β-D-glucosidase from *H. grisea* var. *thermoidea*, with the additional practical advantage of requiring a short time for maximal production.

Cellular distribution of β-D-glucosidase

Around 74% of the β-D-glucosidase activity that was present in the mycelia induced with sugarcane bagasse was bound to the hyphal surface, since it could be detected by assaying the intact mycelium. Part of the enzyme could be extracted by successive washings with chilled water. Even after this procedure, 50% of the total enzyme remained adsorbed to the cell surface, and only 16% was detected after cell disruption, corresponding to the intracellular pool. However, with aging

Table 2. Effects of several carbohydrates on the crude ß-D-glucosidase activity obtained from *H. grisea* var. *thermoidea*

Effector (1%)	ß-D-Glucosidase (U/ml)
Nil (control)	0.60±0.06 (100%)
Arabinose	0.55±0.04 (92%)
Cellobiose	0.19±0.02 (32%)
Fructose	0.62±0.05 (103%)
Trehalose	0.61±0.04 (102%)
Maltose	0.63±0.05 (105%)
Xylose	1.21±0.11 (202%)
Glucose	1.13±0.09 (188%)
Sucrose	0.64±0.06 (107%)
Mannose	0.75±0.08 (125%)

Conidia were inoculated into liquid culture medium supplemented with 1% sugarcane bagasse, and the mycelia were collected after 20 h of growth at 40°C and 145 rpm. Enzymatic activity was assayed in 50 mM sodium phosphate buffer, pH 6.0, at 50°C, using 5 mM pNP-Glc as substrate. Data are the Mean ±SD of six different experiments (n=6).

of the culture, a great amount of ß-glucosidase activity was released into the extracellular medium.

In vitro effects of carbohydrates on the β-D-glucosidase activity of crude enzyme preparations

The effects of several carbohydrates on the β-D-glucosidase activity of crude enzyme preparations obtained from sugarcane bagasse cultures of *H. grisea* var. *thermoidea* are shown in Table 2. Unexpectedly, glucose and xylose exerted a strong stimulatory effect at 1% concentration, increasing the enzymatic activity by about 2 fold. The other carbohydrates tested were without any significant effect, except for cellobiose, the natural substrate of β-D-glucosidases and a potent competitive inhibitor of pNP-Glc hydrolysis.

Some microbial glucose-tolerant β-glucosidases were previously reported, from both fungal (Saha and Bothast, 1996a, 1996b; Riou *et al*., 1998; Sonia *et al*., 2008) and bacterial (Osaki and Yamada, 1991; Harchand and Singh, 1997) sources. Although less common, some glucose-stimulated forms were also described in bacteria (Wrigth *et al*., 1992; Perez-Pons *et al*., 1995) and fungi (Decker *et al*., 2001; Sonia *et al*., 2008). To date, only a single β-glucosidase from the thermophilic fungus *S. thermophilum*, stimulated by either glucose or xylose, has been characterized (Zanoelo *et al*., 2004). However, this enzyme was produced using Avicel as the carbon source, which is a costly substrate compared to sugarcane bagasse. Moreover, the production of ß-glucosidase activity by *S. thermophilum* was about 6-fold lower than that observed in this study for *H. grisea* var. *thermoidea*.

The production of ß-glucosidases by *H. grisea* var. *thermoidea* strains has been previously described in a few reports. However, most forms were strongly inhibited by glucose (Polizeli *et al*., 1996; Peralta *et al*., 1997), whereas in earlier studies (Peralta *et al*., 1990), the effect of glucose or other carbohydrates on the enzyme activity was not investigated.

Naturally occurring cellulosic materials are structurally complex, and the cellulosic fibers are usually surrounded by a matrix of other structural biopolymers, mainly hemicelluloses and lignin (Lynd *et al*., 2002; Kumar *et al*., 2008). Hemicelluloses, representing 20-35% of plant dry weight are, similar to cellulose, a ready source of fermentable sugars, largely xylose, for industrial applications. Acting in concert with endo- and exocellulases and xylanases, glucose- or xylose-stimulated β-glucosidases may be particularly advantageous to accomplish the total saccharification of lignocellulosic materials. Moreover, the stimulation of *H. grisea* var *thermoidea* β-D-glucosidase activity by glucose or xylose in crude preparations undoubtedly constitutes a technical advantage, avoiding expensive and time-consuming purification procedures.

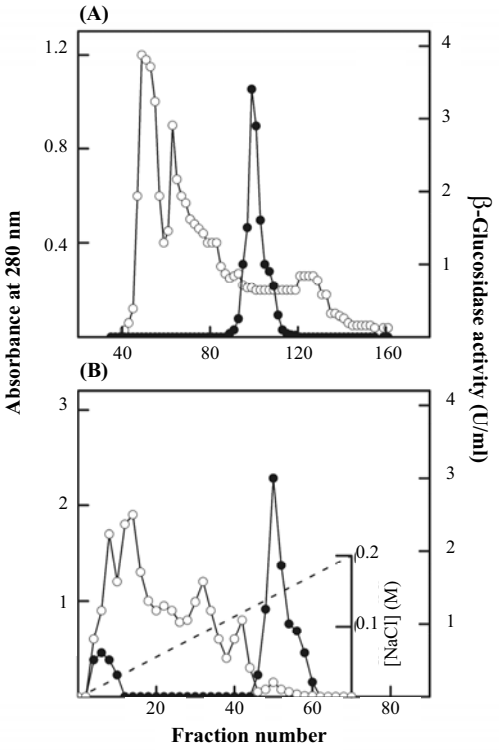

Fig. 1. Chromatographic profiles of *H. grisea* var. *thermoidea* β-D-glucosidase in Sephadex G-200 (A) and DEAE-cellulose (B). After precipitation of the crude enzyme preparation with 75% $(NH_4)_2SO_4$, the precipitate was dialyzed against water, lyophilized, dissolved in 5.0 ml of 50 mM potassium phosphate buffer, pH 6.0 (buffer A), and applied to a Sephadex G-200 column (4.5×40.0 cm), equilibrated and eluted with the same buffer, at a flow rate of 2.5 ml/h. Fractions of 3.0 ml were collected, and those with higher activity were pooled and applied to a DEAE-cellulose column (16×1.5 cm) equilibrated in buffer A and eluted (3.0 ml fractions) with a linear gradient (0-0.2 M) of NaCl in the same buffer, at a flow rate of 18.0 ml/h. Symbols: (●) β-D-glucosidase activity; (○) absorbance at 280 nm.

Enzyme purification and molecular properties

β-D-glucosidase from *H. grisea* var. *thermoidea* was successfully purified through ammonium sulfate precipitation, followed by Sephadex G-200 and DEAE-cellulose chromatography. A single sharp, symmetrical activity peak eluted from the gel-filtration column (Fig. 1A). Ion-exchange chromatography of the pooled active fractions resulted in two activity peaks (Fig. 1B). The peak eluting with 130-150 mM NaCl contained the highest activity against pNP-Glc, and its most active fractions

Table 3. Purification of β-D-glucosidase from *H. grisea* var. *thermoidea* grown in 1% sugarcane bagasse

Step	Total protein (mg)	Total units (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	82.64	243.56	2.95	1.0	100
$(NH_4)_2SO_4$ (80%)	63.07	223.26	3.54	1.2	92
Sephadex G-200	12.61	112.78	8.94	3.0	46
DEAE-cellulose	2.52	82.25	32.39	11.0	33

β-D-Glucosidase activity was estimated at 50°C in 50 mM sodium phosphate buffer, pH 6.0, using 5 mM pNP-Glc as substrate. Data are the mean values of six different experiments (n=6).

were pooled, dialyzed, lyophilized, and used for further experiments.

Data of a typical enzyme purification procedure are summarized in Table 3. At the final step, a specific activity of 32.39 U/mg was achieved, with a yield of 33% and an 11-fold purification. In comparison, in spite of a similar yield, the specific activity obtained for the mycelial ß-glucosidase purified from *H. grisea* var. *thermoidea* grown in Avicel was about 2-fold lower (Peralta *et al*., 1990).

The homogeneity of the purified enzyme was confirmed by nondenaturing PAGE analysis, which showed a single protein band after staining with Coomassie blue (Fig. 2A), coincident with a β-D-glucosidase activity band detected using 6-bromo-2-naphthyl-β-D-glucopyranoside as the substrate (Fig. 2B). A single protein band was also detected after electrophoresis under denaturing conditions (SDS-PAGE), corresponding to an apparent molecular mass of 57 kDa (Fig. 2C). The similar molecular mass, 60 kDa, determined by gel-filtration analysis suggested that the purified enzyme is a monomer, in concordance with previous reports regarding β-D-glucosidases from *H. grisea* var *thermoidea* (Peralta *et al*., 1990, 1997).

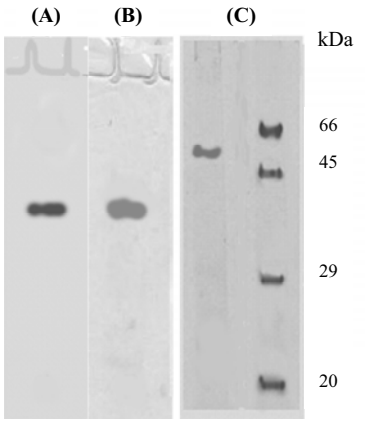

Fig. 2. Analysis of *H. grisea* var. *thermoidea* β-D-glucosidase by PAGE (A and B) and SDS-PAGE (C) using 25 μg protein per lane. Lanes (A) and (C), Coomassie blue staining for protein; (B), activity test for β-D-glucosidase activity, using 6-bromo-2-naphthyl-β-D-glucopyranoside. Other details as described in the 'Materials and Methods' section.

Monomeric β-D-glucosidases with apparent molecular masses ranging from 40 to 50 kDa were described in *H. insolens* (Rao and Murthy, 1988), *S. thermophilum* (Zanoelo *et al*., 2004), *Talaromyces thermophilus* (Nakkharat and Haltrich, 2006), and *C. thermophilum* var. *coprophilum* (Venturi *et al*., 2002). In contrast, dimeric enzymes with native molecular masses of about 200 kDa were found in *Paecilomyces thermophila* (Yang *et al*., 2008) and *Thermomyces lanuginosus* (Lin *et al*., 1999). Apparent molecular masses ranging from 40 to 250 kDa were reported for β-D-glucosidases from other fungal sources (Hayashida *et al*., 1988; Masheshwari *et al*., 2000; Bhatia *et al*., 2002; Yoon *et al*., 2008).

Nearly all β-D-glucosidases are glycoproteins (Masheshwari *et al*., 2000), and thermophilic fungi usually produce enzymes with elevated carbohydrate content, particularly those secreted into the extracellular medium (Peralta *et al*., 1997; Venturi *et al*., 2002; Yang *et al*., 2008). The β-D-glucosidase from *H. grisea* var. *thermoidea* showed a carbohydrate content of about 43%, elevated when compared to the contents estimated for the intracellular enzymes from *H. insolens* (Hayashida *et al*., 1988; Rao and Murthy, 1988) and *S. thermophilum* (Zanoelo *et al*., 2004). Interestingly, a 2-fold-lower value was determined

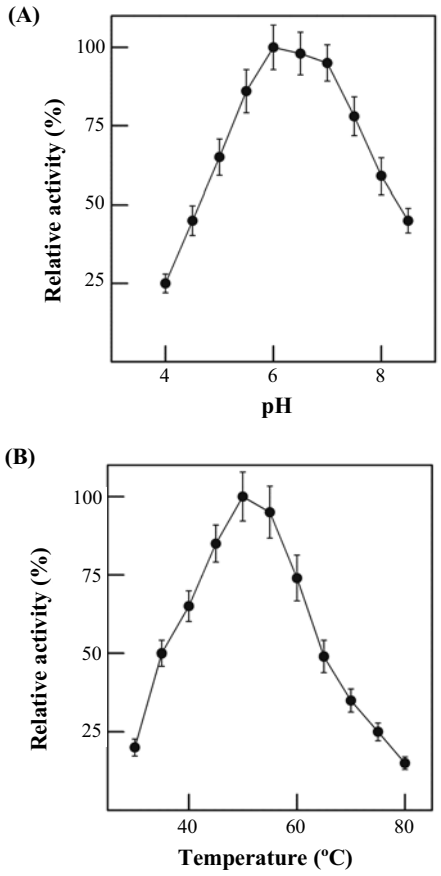

Fig. 3. Optimal pH (A) and temperature (B) of the purified β-D-glucosidase obtained from *H. grisea* var. *thermoidea*. One hundred percent specific activity corresponded to 31.5±1.9 U/mg. The buffers used, at 50 mM concentration, were sodium acetate (pH 4.0-5.5), sodium phosphate (pH 6.0-8.0), and glycine (pH 8.5). The values shown represent the mean values from triplicate experiments (n=3).

for the intracellular enzyme purified from the same strain of *H. grisea* var. *thermoidea* grown in Avicel (Peralta *et al*., 1990). Influence of the carbon source on the glycosylation degree has been previously reported for *T. reesei* cellobiohydrolase I (Klarskov *et al*., 1997; Maras *et al*., 1997; Harrison *et al*., 1998; Hui *et al*., 2001; Stals *et al*., 2004a, 2004b), *Saccharomyces cerevisiae* phosphoglucomutase (Dey *et al*., 1994), and *A. versicolor* ß-xylosidases (Andrade *et al*., 2004; Somera *et al*., 2009).

Effects of temperature and pH on catalytic activity

The activity of the purified β-D-glucosidase progressively increased from pH 4.0 to 6.0, reaching a plateau at pH values ranging from 6.0-7.0 (Fig. 3A). A gradual decrease occurred at pH values higher than 7.0, with a residual activity of about 45% being detected at pH 8.5. Commonly, fungal β-D-glucosidases show optimal pH in the range 4.0 to 6.5 (Masheshwari *et al*., 2000; Bhatia *et al*., 2002; Bhiri *et al*., 2008; Yang *et al*., 2008; Yoon *et al*., 2008).

The purified enzyme showed maximal activity at 50°C, which was almost unaltered at 55°C (Fig. 3B). Above 60°C, the activity progressively decreased, reaching a residual activity of only 15% of the maximal value at 80°C. The enzyme was thermostable up to 60 min when incubated in water at 55°C. In contrast, at 55°C, half-lives of about 12 min and 20 min were obtained for the purified intracellular β-D-glucosidases from *H. grisea* var. *thermoidea* and *S. thermophilum*, respectively, grown on Avicel (Peralta *et al*., 1990; Zanoelo *et al*., 2004). The higher thermal stability of the enzyme produced by *H. grisea* when sugarcane bagasse was the carbon source may be tentatively attributed to its higher sugar content. In fact, several authors have attributed a protective effect against denaturation and proteolytic attack to the glycosylation of enzymes (Kern *et al*., 1992; Varki, 1993; Meldgaard and Svendsen, 1994; Lige *et al*., 2001; Venturi *et al*., 2002).

When incubated at 60°C, the purified β-D-glucosidase showed a half-life of 7 min (Fig. 4). The presence of glucose (50 mM) protected the enzyme from inactivation, increasing the half-life to 14 min. Xylose and sucrose also slightly improved the thermal stability of the purified enzyme (Fig. 4). β-D-Glucosidases from thermophilic fungi show temperature optima ranging from 55°C to 75°C, usually higher than those observed for enzymes from mesophilic organisms (Peralta *et al*., 1997; Riou *et al*., 1998; Masheshwari *et al*., 2000; Venturi *et al*., 2002; Kaur *et al*., 2007; Yang *et al*., 2008). Amazingly, a ß-glucosidase recently purified from the basidiomycete *Fomitopsis palustris* grown in Avicel showed a half-life of 15 h at 65°C (Yoon *et al*., 2008). Compared to the glucose- or xylose-stimulated β-D-glucosidase from *S. thermophilum* (Zanoelo *et al*., 2004), the enzyme purified from *H. grisea* var. *thermoidea* showed similar optimal temperature but higher thermal stability. Moreover, the stabilizing effect of glucose against thermal inactivation confers to the *H. grisea* enzyme an additional attractive feature for biotechnological applications.

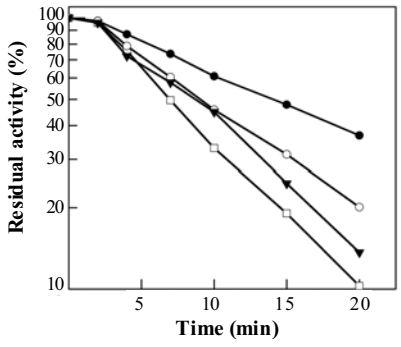

Fig. 4. Effects of different carbohydrates on the thermal stability of the purified β-D-glucosidase obtained from *H. grisea* var. *thermoidea* at 60°C. Samples of the purified enzyme were incubated in aqueous solutions either containing 50 mM glucose (●), xylose (○), or sucrose (▲), or in the absence of free carbohydrates (□). One hundred percent specific activity corresponded to 30.7±2.0 U/mg. The values shown represent the mean values from triplicate experiments (n=3).

Table 4. Substrate specificity of purified ß-D-glucosidase[a]

Substrate[b]	Specific activity[c] (U/mg)
pNP-Glc	29.49±3.23
pNP-Gal	13.29±1.47
oNP-Gal	43.31±3.48
pNP-Fuc	64.72±4.23
pNP-Xyl	5.25±0.79
pNP-α-Glc	ND
pNP-α-Gal	ND
Cellobiose	20.12±2.14
Lactose	11.22±1.46
Avicel	ND
CM-cellulose (medium viscosity)	ND
Xylan	ND
Starch	ND
Pectin	ND

[a] Hydrolytic activities on different substrates were estimated in 50 mM sodium phosphate buffer, pH 6.0, at 50°C.
[b] Artificial substrates were used at a final concentration of 5 mM, cellobiose and lactose at 10 mM, and polysaccharides at 1% (w/v).
[c] Data are the mean values±SD of four different experiments (n=4).
ND, activity not detected by the method used even after 20 h of reaction

Substrate specificity and kinetic parameters of the purified β-D-glucosidase

H. grisea var. *thermoidea* β-D-glucosidase showed broad substrate specificity (Table 4), hydrolyzing cellobiose, lactose, and various aryl-β-glycosides (pNP-Glc, pNP-Gal, oNP-Gal, pNP-Fuc, pNP-Xyl), but not aryl-α-glycosides (pNP-α-Glc and pNP-α-Gal), thereby suggesting a high specificity for the β-anomeric configuration of the mono- or disaccharide but not for glucose. The enzyme was also unable to degrade polymeric substrates. Broad substrate specificity is an ordinary attribute of fungal β-D-glucosidases (Riou *et al*., 1998; Parry *et al*., 2001; Bhatia *et al*., 2002; Zanoelo *et al*., 2004; Nakkarat and Haltrich, 2006; Karnchanatat *et al*., 2007; Yang *et al*., 2008).

The synthetic substrates pNP-Fuc (V_M=128.26±12.83 U/mg), oNP-Gal (V_M=54.58±7.60 U/mg), and pNP-Glc (V_M=41.33±4.90 U/mg) were hydrolyzed by the purified enzyme with the highest specific activities (Table 5). The enzyme also showed high apparent affinities for pNP-Fuc (K_M=0.05±0.005 mM)

Table 5. Apparent kinetic parameters for the hydrolysis of several substrates by the purified ß-D-glucosidase

Substrate	K_M (mM)	V_M (U/mg)	V_M/K_M
pNP-Glc (0.01-2.0 mM)	0.12±0.01	41.33±4.90	344.40
pNP-Gal (0.1-30 mM)	1.32±0.18	26.13±3.66	19.80
oNP-Gal (0.1-40 mM)	6.80±1.02	54.58±7.60	8.03
pNP-Fuc (0.005-2 mM)	0.05±0.005	128.26±12.83	2565.20
Cellobiose (0.01-5 mM)	0.27±0.03	28.73±2.87	106.41
Lactose (0.1-20 mM)	1.42±0.17	12.17±1.82	8.57

Hydrolytic activity on different substrates was assayed at 50°C in 50 mM sodium phosphate buffer, pH 6.0, at varying substrate concentrations in the ranges indicated. Data are the Mean±SD of four different experiments (n=4).

and pNPGlc (K_M=0.12±0.01 mM). Greater maximal velocities and affinities for synthetic substrates rather than natural ones are kinetic features shared by β-D-glucosidases from several fungal sources (Hayashida *et al*., 1988; Venturi *et al*., 2002; Zanoelo *et al*., 2004; Yang *et al*., 2008; Yoon *et al*., 2008).

Among the natural substrates, the *H. grisea* var. *thermoidea* enzyme hydrolyzed cellobiose with substantially higher specific activity (V_M=28.73±2.87 U/mg) and apparent affinity (K_M=0.27±0.03 mM), compared to lactose (V_M=12.17±1.82 U/mg; K_M=1.42±0.17 mM), and, as evaluated by the V_M/K_M ratio, cellobiose was hydrolyzed 12-fold more efficiently (Table 5). Interestingly, the enzyme showed 6- and 7-fold, respectively, higher apparent affinity and specific activity for cellobiose, compared to the glucose- or xylose-stimulated β-D-glucosidase from *S. thermophilum* (Zanoelo *et al*., 2004), resulting in a V_M/K_M ratio about 42-fold higher.

Finally, the β-D-glucosidase purified from the same strain of *H. grisea* var. *thermoidea* grown on Avicel showed 2-fold lower specific activity and apparent affinity for cellobiose, resulting in a 4-fold lower catalytic efficiency (Peralta *et al*., 1990). Thus, in addition to the advantage of a lower cost, the use of sugarcane bagasse as a carbon source in the culture medium resulted in the production of a β-glucosidase with better catalytic properties, considering the hydrolysis of cellobiose and, ultimately, cellulose saccharification.

Effects of carbohydrates on purified β-D-glucosidase activity

The effects of carbohydrates on the activity of the purified enzyme were similar to those previously observed for crude enzyme preparations (Table 2), including the strong stimulatory effects of glucose and xylose. These results point out that the stimulation by glucose or xylose is an intrinsic property of this particular β-D-glucosidase, not dependent on the presence of any other component of the crude extract.

The maximal stimulation of the purified enzyme activity by glucose (2.2-fold) or xylose (2-fold) when pNP-Glc was the substrate occurred in the range 100-175 mM (Fig. 5A), and was followed by a gradual inhibition at higher concentrations. However, the activity remained higher than that determined in the absence of activators up to 500 mM xylose or glucose. Synergistic effects of monosaccharides on enzyme activation were not observed, suggesting that they may bind to a common site in the enzyme molecule (Fig. 5A). Importantly, xylose was also a potent stimulator of the hydrolysis of natural substrates by the purified enzyme (Fig. 5B). Increasing

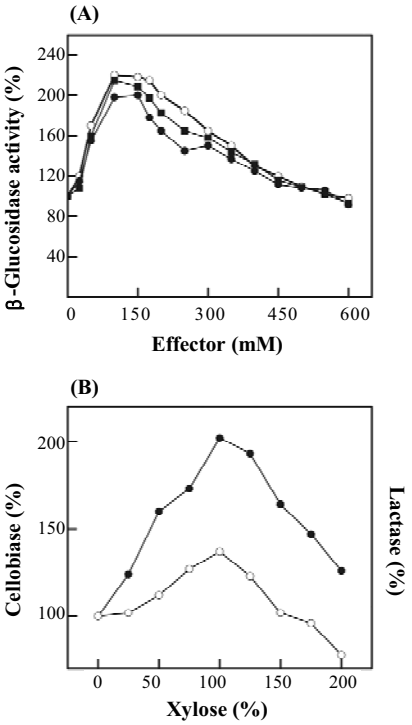

Fig. 5. Stimulatory effect of glucose and/or xylose on the activity of the purified β-D-glucosidase obtained from *H. grisea* var. *thermoidea*. (A) Hydrolytic activity on 5 mM pNP-Glc in the presence of increasing concentrations of (○) glucose, (●) xylose, or (■) equimolar mixtures of glucose and xylose, containing each sugar at a concentration equal to that indicated in the abscissa. (B) Hydrolytic activity on 10 mM (●) cellobiose or (○) lactose in the presence of increasing concentrations of xylose. One hundred percent activities correspond to the following: pNP-Glucosidase, 31.02±2.03 U/mg; cellobiase, 23.73±1.76 U/mg; and lactase, 26.45±1.89 U/mg.

concentrations of xylose up to 100 mM increased the cellobiase activity 2-fold, whereas a 1.5-fold increase was observed for the lactase activity. Amazingly, cellobiase activity was higher than that of the control even at xylose concentrations as high as 200 mM (Fig. 5B). A comparable stimulatory effect of glucose on cellobiase activity (2.1-fold) was detected using HPLC. Similar results were described by Zanoelo *et al*. (2004) for the glucose- or xylose-stimulated β-D-glucosidase from *S. thermophilum*.

The stimulatory effect of glucose on *H. grisea* var. *thermoidea* purified enzyme activity at concentrations up to 175 mM may be attributed to its binding to a monosaccharide-specific site, possibly inducing a conformational change at the active site that results in increased hydrolysis rate. As the glucose concentration increases, however, a competition between glucose and the substrate for the active site may simultaneously occur, leading to a gradual decrease in enzyme activity. Finally, at glucose concentrations as high as 500-600 mM, the competition for the active site may result in a specific activity lower than that observed in the absence of the monosaccharide. A similar interpretation should explain the effects of xylose.

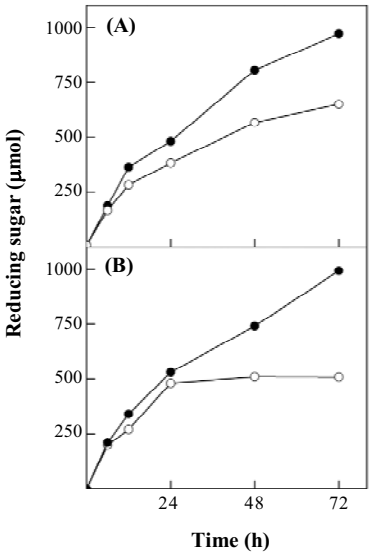

Fig. 6. Progress kinetics of the enzymatic hydrolysis of sugarcane bagasse. Hydrolysis was carried out at 50°C at 100 rpm in 50 mM sodium acetate buffer, pH 5.0, containing 6 mg/ml sugarcane bagasse and 2 mM sodium azide. (A) The reaction was carried out with (○) 10 FPU cellulase activity from *T. reesei* mutant RP-98; (●) a mixture of 10 FPU cellulase activity from *T. reesei* and 0.1 U of purified *H. grisea* var. *thermoidea* β-glucosidase. (B) The reaction was carried out with (○) 10 FPU cellulase activity from *T. reesei* mutant RP-98; (●) a mixture of 10 FPU cellulase activity from *T. reesei* and an aliquot of a crude *H. grisea* var. *thermoidea* enzyme preparation containing 0.1 U β-glucosidase activity.

Synergism between *H. grisea* var. *thermoidea* β-glucosidase and *T. reesei* cellulases in sugarcane bagasse hydrolysis

The effectiveness in sugarcane bagasse hydrolysis brought about by a mixture of the purified β-glucosidase and an extracellular cellulase-rich crude extract from a *T. reesei* mutant RP-98, a cellulase hyperproducer strain, was investigated (Fig. 6). Remarkably, after 72 h of reaction, the amount of reducing sugars released increased by about 50% in the presence of 0.1 U of β-glucosidase activity, when compared to that obtained using 10 FPU cellulase activity from *T. reesei* only (Fig. 6A). Importantly, similar results were obtained when the purified enzyme was substituted by *H. grisea* var. *thermoidea* crude-enzyme preparations (Fig. 6B).

Filamentous fungi are good sources of cellulases and hemicellulases, and mutant strains of *Trichoderma* sp. have long been considered the best producers of cellulases (Nieves *et al*., 1998; Galbe and Zacchi, 2002). However, the main drawback with reference to *Trichoderma* is its low level of β-glucosidase activity (Duff and Murray, 1996; Nieves *et al*., 1998), which limits cellobiose conversion to glucose, leading to the inhibition of the cellulose hydrolysis process as a whole. The pronounced synergism observed between *T. reesei* cellulases and *H. grisea* var. *thermoidea* β-glucosidase (Fig. 6), in association with the amazing properties of this enzyme in terms of stimulation by glucose and xylose, high thermal stability, low cost of production, and increased release into the culture medium with the aging of the mycelium, suggest that it could be very useful for the development of a multienzyme mixture with high performance for the biodegradation of lignocellulosic materials.

Finally, ß-glucosidases that are activated by glucose may constitute a new class of cellobiases, eventually characteristic of thermophilic molds.

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