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Production of Cellulases by *Aspergillus fumigatus* and Characterization of One β-Glucosidase

Eduardo A. Ximenes, Carlos R. Felix, Cirano J. Ulhoa

Departamento de Biologia, 70.910.900 Universidade de Brasília, Brasília, DF, Brasil

Abstract. *Aspergillus fumigatus* produces substantial extracellular cellulases on several cellulosic substrates including simple sugars. Low glucose potentiates enzyme production, but most cellulose-induced cellulases are repressed by high glucose. As production of cellulase in a wide substrate range is unusual, the cellulolytic complex of this thermophilic fungus was investigated. A β-glucosidase was separated by gel filtration and ion-exchange chromatography. It migrated in native polyacrylamide gel as a single protein (130 kDa), which split under denaturing conditions into two smaller proteins having molecular masses of 90 kDa and 45 kDa. However, only the 90-kDa protein was active. Conventional chromatographic procedures were unsuccessful for the separation of these two proteins. Therefore, the 130-kDa protein was studied for its kinetic properties. It hydrolyzed *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) and cellobiose, but not β-glucans, laminarin, and *p*-nitrophenyl-β-D-xilopyranoside. The optimal pH and temperature of *p*-NPG and cellobiose hydrolysis were 5.0 and 4.0, and 65°C and 60°C, respectively. The K_m values, determined for cellobiose and *p*-NPG of hydrolysis, were 0.075 mM and 1.36 mM, respectively. Glucose competitively inhibited the hydrolysis of *p*-NPG. The K_i was 3.5 mM.

Cellulose may be hydrolyzed enzymatically by the combined action of at least three enzymes: exoglucanases (EC 3.2.1.91, 1,4-β-D-glucan-cellobiohydrolase), endocellulases (EC 3.2.1.4, endocellulase) and β-glucosidases (EC 3.2.1.21). Endocellulases attack randomly internal glucosidic bonds of cellulose chains, thereby producing polymer chain ends and soluble oligosaccharides. Celobiohydrolases cleave cellobiosyl residues from the ends of cellulose chains. β-Glucosidases catalyze the hydrolysis of cellobiose, which is inhibitory to the exo- and endocellulases [6].

Among fungi, *Trichoderma reesei*, a filamentous mesophilic fungus and mutants derived from it, are the best known cellulase producers. Its cellulolytic complex has been investigated thoroughly [16], and it is considered to show commercial potential. The low β-glucosidase activity in the secreted enzyme complex, however, seems to be the rate-limiting factor if glucose is the desired product [24]. In addition, the major problem of utilizing the enzymes of mesophilic organisms for the production of fermentable substrates is the low rate of hydrolysis achieved at the optimal growth temperature of the organism and the relatively poor temperature stability of the enzymes [7]. When compared with mesophilic fungi, thermophilic fungi have higher growth and cellulase production rates [7].

Aspergillus fumigatus secretes several glycosidases in culture media [15, 20, 21]. A strain of *A. fumigatus* isolated from a hot water fountain in Brazil grew strongly on a variety of amylaceous and lignocellulosic substrates. Here, we report on the production of cellulases by this isolate and on the purification of one of its enzymes that exhibited aryl-β-glucosidase and cellobiase activity.

Materials and Methods

Growth conditions. *Aspergillus fumigatus* was isolated from a hot water fountain in Brazil (Caldas Novas-GO) and cultured in MYG medium (0.2% malt extract, 0.2% yeast extract, 2% glucose, and 2% agar) at 42°C. Mycelium used to study induction of enzyme production was first grown in Pontecorvo liquid medium [17] with glucose as the carbon source, and inoculated with 10 ml of a conidial suspension containing 10^6 conidia per ml. After 48 h of growth at 42°C, the mycelium was washed four times with 0.9% NaCl and resuspended in fresh medium [17]. Samples (10 ml) of

Correspondence to: E.A. Ximenes

Table 1. Cellulase activity in the culture fluid of *A. fumigatus* growing in different carbon sources

Carbon sources	FPAase (IU/ml) × 10^2	ρ-NPGase (IU/ml) × 10^2	Cellobiase (IU/ml) × 10^3
Glucose	5.0	2.5	6.7
Manose	3.9	2.0	4.4
Xylose	5.0	1.0	1.7
Lactose	12.8	1.6	2.8
Cellobiose	6.7	5.2	6.7
Xylan	6.7	1.7	5.0
Raffinose	3.9	3.1	1.1
Avicel	5.5	1.6	2.2
Wheat straw	8.9	10.4	10.5
CM-cellulose	7.2	2.5	3.9
Filter-paper	15.5	6.8	15.5
Cotton	4.4	1.1	2.8

The activity values are the means of two different experiments. In each experiment, enzyme assays were run in triplicates.

the mycelial suspension (2.3 mg on dry-weight basis) were then transferred to 15 ml of the same medium with or without glucose and/or other substrates. The cultures were then incubated on a rotary shaker at 42°C and 120 rpm.

The crude enzyme used for purification was produced in 2.5 L of liquid medium [0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$] with ground filter paper (0.5%) as the carbon source and inoculated with 30 ml of a conidial suspension (10^6 conidia/ml). The cultures were incubated at 42°C and 120 rpm, for 96 h. The culture supernatants were then collected through filtration on filter paper and stored at 4°C, after addition of NaN_3 (0.02%).

Enzyme assays. The activity against filter paper (FPAase activity) was assayed by measuring the production of reducing sugar (RS) by the DNS method [14]. The reaction mixture contained 0.5 ml of 0.05 M sodium citrate (pH 4.5), one strip of filter paper (1 × 6 cm), and an appropriate volume (0.05–0.25 ml) of enzyme. The reactions were run for 60 min at 50°C and stopped by the addition of the DNS reagent. One enzyme unit (IU) corresponded to the amount of enzyme that produces 1 µmol of glucose equivalent per min.

Aryl-β-glucosidase activity was assayed by measuring at 405 nm the liberation of *p*-nitrophenol (*p*-NP) from *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG). The reaction mixture contained 0.5 ml of 0.1 M sodium acetate (pH 5.0), 0.25 ml of 4 mM *p*-NPG, 0.1–0.25 ml of enzyme, and water to a final volume of 1 ml. The reaction was carried out for 10 min at 42°C (except when stated) and stopped by the addition of 1 ml of 1 M sodium carbonate. One unit (IU) was defined as the amount of enzyme that produced 1 µmol of *p*-NP per min.

Cellobiase activity was assayed by measuring the production of glucose from cellobiose by the glucose-oxidase method [1]. The reaction mixture contained 1 ml of 0.05 M sodium acetate (pH 5.0), 5 mg of cellobiose, and 1 ml of enzyme solution. The reaction was allowed to proceed for 30 min at 50°C and stopped by placing the tubes in boiling water. One enzyme unit (IU) was defined as the amount of enzyme that produced 1 µmol of glucose per min.

Purification of β-glucosidase. The crude enzyme was concentrated by ultrafiltration on an Amicon membrane (PM 10) and passed through a Sephacryl S-200 column (3 cm × 92 cm) equilibrated and eluted with 0.01 M sodium acetate (pH 5.0). Samples of 5 ml were collected at a flow rate of 16 ml/h. The active protein fractions were pooled, concentrated by ultrafiltration, and loaded on an S-Sepharose column (2 cm × 42 cm) equilibrated with 0.01 M sodium acetate (pH 5.0). Elution was carried out with 100 ml of acetate buffer followed by a linear gradient formed with 150 ml of 0.01 M sodium acetate (pH 5.0) and 150 ml of the same buffer containing 0.5 M NaCl.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis under denaturing conditions was carried out by the method of Laemmli [12], and under non-denaturing conditions according to the Sigma method (Non-denaturing protein molecular weight marker kit, October 1986). Proteins were silver stained as recommended by Blum et al. [2]. β-glucosidase activity in native gel was revealed by the method of Rutemberg et al. [22], with 6-bromo-naphthyl-β-D-glucopyranoside as the substrate. For determination of enzyme activity in gels containing SDS, gels were cut longitudinally into 2-mm pieces, and the slices homogenized in 0.5 ml of 0.1 M acetate buffer, pH 5.0. *p*-NPG was added to the homogenate and the reaction carried out as described for aryl-β-glucosidase assay.

Protein determination. Protein was quantified by the Coomassie blue method [3] with bovine serum albumin as the standard.

Results and Discussion

Production of cellulases. Substantial quantities of FPAse, aryl-β-glucosidase, and cellobiase are produced by *A. fumigatus* cultured on simple sugars and several cellulosic substrates (Table 1). Whereas the highest FPAse activity was induced by filter paper and lactose, the highest aryl-β-D-glucosidase was induced by filter paper and wheat straw. Filter paper was the most effective substrate to induce cellobiase. Although reported before for *Thermoascus aurantiacus* [7], it is rare that an organism produces cellulolytic enzymes on so wide a substrate range. In general, cellulosic materials act as inducers, and readily metabolized carbon compounds as repressors [18]. Cellulolytic fungi, such as *T. reesei*, grow on a wide variety of carbon sources, but cellulase is produced only in the presence of cellulose and cellodextrins, including cellobiose. In a few cases, lactose and sophorose are also inducers [13]. In *Sporotrichum thermophile*, low activity is detected on a variety of low-molecular-weight substrates even though the substrates utilized are not equivalent for supporting the growth of the organism [4]. The capacity of *A. fumigatus* to produce cellulases was not compared with other well-known cellulolytic fungi such as *T. reesei* [16]. The ability of *A. fumigatus*, however, to grow on several substrates and produce hydrolytic enzymes of economic value, together with the properties of its β-glucosidase reported here, encourages further investigations.

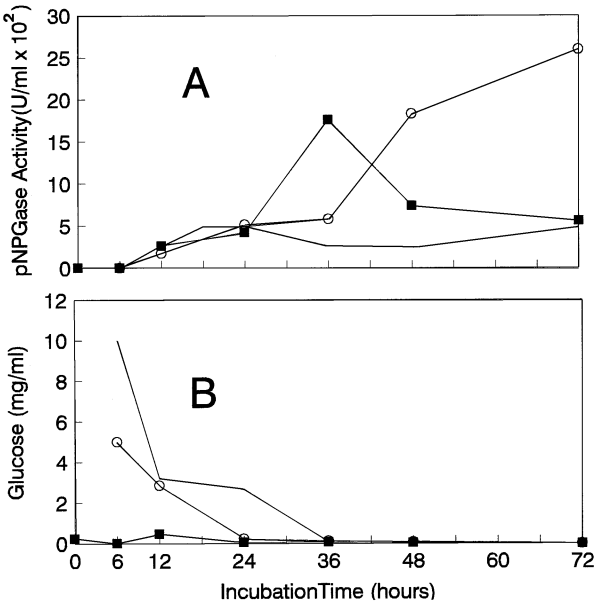

Fig. 1. (A) Time course of β-glucosidase production by *A. fumigatus* growing in filter paper-containing medium without (■) or with 0.5% (○) and 1% (—) glucose; (B) amount of glucose in the culture fluid.

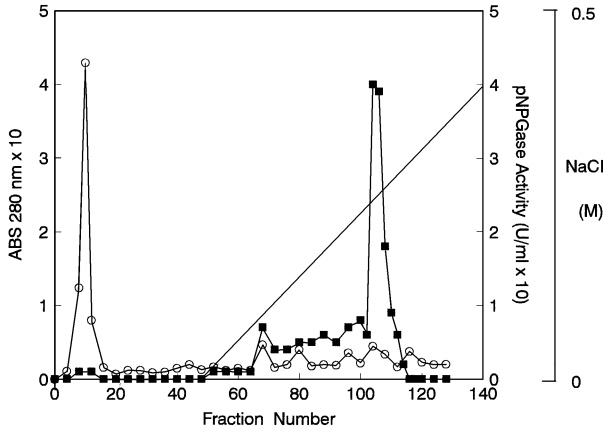

Fig. 2. Ion-exchange profile of the β-glucosidase on an S-Sepharose column. Absorbance at 280 nm (○); aryl-β-glucosidase activity (■); NaCl concentration (—).

Table 2. Summary of the purification steps of the β-glucosidase produced by *A. fumigatus*

Steps	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Yield (%)
Crude enzyme	27.0	33.10	0.81	100.0
Sepharcyl eluate	5.0	4.30	1.16	18.50
Sepharose eluate	1.8	0.08	22.50	6.70

Aryl-β-glucosidase induction. The aryl-β-D-glucosidase activity induced by filter paper raised and decreased within 36 h. The addition of 0.5% glucose to the medium containing filter paper increased the enzyme activity substantially. This activity, however, was drastically reduced by 1.0% glucose (Fig. 1A). Although mycelia growth was not monitored, cell mass increase could not account for the higher enzyme activity in the culture containing filter paper and 0.5% glucose. In that case, the activity in the culture containing 1% glucose should have been at least equivalent to the 0.5% glucose-containing culture. The *A. fumigatus* aryl-β-glucosidase was shown to be inhibited by glucose (Table 3). Nevertheless, enzyme inhibition can not explain the lack of activity in the medium containing 1% glucose, since the exogenous glucose added was consumed in any case within the same time period (Fig. 1B). Since the enzyme activity rose from a low level only after the glucose was exhausted (at 36 h), it is likely that production of the cellulases by *A. fumigatus* was accomplished by (i) a constitutive system that produced a basal level and (ii) an induced mechanism that synthesized most of the cellulases and was repressed by the end products.

Aryl-β-glucosidase purification. Gel filtration chromatography of the crude enzyme gave several protein fractions. Only two fractions, however, showed aryl-β-D-glucosidase activity (not shown). S-Sepharose chromatography of the major aryl-β-D-glucosidase fraction from Sephacryl chromatography (peak I) resulted in a major protein fraction that was eluted with 0.3 M NaCl (Fig. 2). Table 2 summarizes the data of a typical purification of the extracellular aryl-β-D-glucosidase. The enzyme was purified about 27-fold to a specific activity of 22.5 U/mg of protein and a yield of 6.7%.

Native PAGE of the enzyme, resulting from the two chromatographic procedures, revealed a single protein with a molecular mass of about 130 kDa (Fig. 3A). Its migration profile coincided with the enzyme activity against the synthetic substrate 6-bromo-naphthyl-β-D-glucopyranoside (not shown). Nevertheless, in gels containing SDS or SDS and urea, the 130-kDa protein split into two polypeptide chains having molecular masses of 90 kDa and 45 kDa (Fig. 3B). Although no activity was observed for the 45-kDa protein, aryl-β-D-glucosidase activity in the SDS-containing gel (not shown) coincided with the 90-kDa protein. It is unlikely, however, that the 45-kDa protein was a contaminant. These two proteins are tightly bound and could not be separated by additional chromatography. The addition of SDS to the eluting

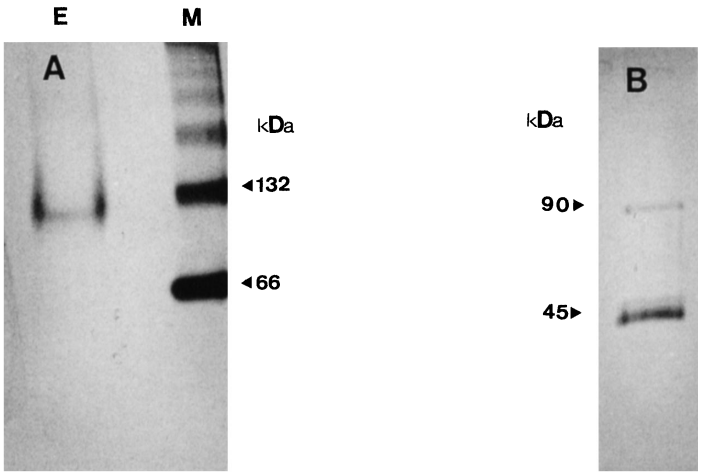

Fig. 3. Native PAGE (A) and electrophoretic analysis on a polyacrylamide gel containing SDS and urea (B) of the *A. fumigatus* β-glucosidase isolated by the Sephacryl S-200 and S-Sepharose chromatographic procedures. Symbols: E, enzyme; M, molecular weight standards.

Table 3. Kinetic parameters for hydrolysis of *ρ*-NPG (at 65°C and pH 5.0) and cellobiose (at 60°C and pH 4.0) by *A. fumigatus* β-glucosidase

Substrates	Range tested	K_M (mM)	Vmax (μmol/min)	Ki (mM)	Optimum temp.	Optimum pH
ρ-NPG	0.05–2.0	0.075	0.55	3.5	65°C	5.0
cellobiose	0.50–15.0	1.36	0.05	—	60°C	4.0

K_m was calculated by the Lineweaver-Burk plot. V_{max} corresponds to μmoles of *ρ*-NP or glucose formed per minute. K_i represents the inhibition constant by glucose.

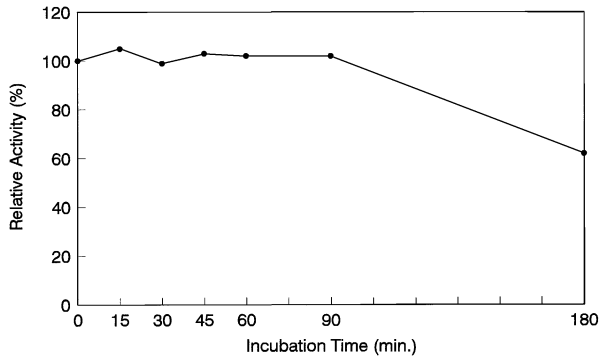

Fig. 4. Effect of temperature on the stability of the β-glucosidase. Sp. act. corresponds to units of aryl-β-D-glucosidase per mg of protein.

buffer resulted in active fractions and turned out to be inconclusive upon SDS-PAGE analysis. Another strain of *A. fumigatus* was reported to produce two β-glucosidases with molecular masses of 340 kDa and 40 kDa [20, 21]. The high-molecular-mass enzyme purified by those authors gave two polypeptide chains in polyacrylamide gel electrophoresis. Additional investigations are needed to clarify the possibility that the 45-kDa protein has any effect on the catalytic property of the 95-kDa protein.

Kinetic determinations. The kinetic properties of the 130-kDa β-glucosidase from *A. fumigatus* were investigated. The optimal pH for hydrolysis of *p*-NPG and cellobiose was 5.0 and 4.0, respectively (Table 3). They agreed with the optimal pH reported for several other fungal β-glucosidases [5, 8, 11], including other *Aspergillus* species [9, 10, 25]. At the optimal pH, the activity against these two substrates was maximal at the temperatures of 65°C (*p*-NPG) and 60°C (cellobiose; Table 3). This enzyme is highly thermostable. At 65°C, it retained 100% and 60% respectively, of its original activity for 1.5 h and 3 h (Fig. 4).

The V_{max} and K_m constants were calculated from the initial rate of *p*-NPG and cellobiose hydrolysis. While *p*-NPG was hydrolyzed with a V_{max} of 0.550 μmol of *p*-NP formed per minute and a K_m of 0.075 mM, cellobiose was hydrolyzed with a V_{max} of 0.050 μmol of glucose formed per minute and a K_m of 1.36 mM (Table 3). These K_m values are equivalent to those of the β-glucosidase produced by *Humicola grisea* [8], *T. reesei* [5], *Thermotoga* sp [23], and *A. niger* [10, 25].

The *A. fumigatus* aryl-β-glucosidase showed very low sensitivity to glucono-1,5-lactone, a specific inhibitor of β-glucosidases (Table 4). The concentration (2 mM) of glucono-lactone, which reduced the enzyme activity to 60%, was 40-fold higher than the concentration (0.05 mM) required to reduce to half the activity of most β-glucosidases [19]. Hydrolysis of *p*-NPG was inhibited by glucose at a concentration range from 0.25 mM to 50 mM, and the corresponding K_i was 3.5 mM (Table 3). This value is considerably higher (2.4-

Table 4. Effect of ions, denaturing compounds, and SDS on the activity of the *A. fumigatus* β-glucosidase

Reagents added (2 mM)	Inhibition (%)
$CaCl_2$	11
$CuSO_4$	19
$CoCl_2$	27
KCl	31
NaCl	32
$HgCl_2$	46
DTE	19
β-mercaptoethanol	20
SDS	24

to 4-fold) than the K_i reported for inhibition of two β-glucosidases from *H. grisea* by glucose [8]. Metal ions, reducing agents, and detergents were weakly effective in inhibiting the 130-kDa β-glucosidase form (Table 4). At a concentration of 2 mM, $HgCl_2$ was the most effective inhibitor. While several thermophilic enzymes are positively modulated by high ionic strength [11], the *A. fumigatus* β-glucosidase was slightly negatively modulated by NaCl (Table 4). In addition, the very low sensitivity of this enzyme to SDS is also noteworthy (Table 4). This property may further help in the separation of the 90-kDa from the 45-kDa protein.

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