Appl Biochem Biotechnol (2008) 149:169–182
DOI 10.1007/s12010-008-8146-y

Purification and Biochemical Characterization of Extracellular β-Glucosidases from the Hypercellulolytic Pol6 Mutant of *Penicillium occitanis*

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Received: 13 August 2007 / Accepted: 19 December 2007 /
Published online: 4 March 2008

Abstract The Pol6 mutant of *Penicillium occitanis* fungus is of great biotechnological interest since it possesses a high capacity of cellulases and β-glucosidase production with high cellulose degradation efficiency (Jain et al., *Enzyme Microb Technol*, 12:691–696, 1990; Hadj-Taieb et al., *Appl Microbiol Biotechnol*, 37:197–201, 1992; Ellouz Chaabouni et al., *Enzyme Microb Technol*, 16:538–542, 1994; Ellouz Chaabouni et al., *Appl Microbiol Biotechnol*, 43:267–269, 1995). In this work, two forms of β-glucosidase (β-glu 1 and β-glu 2) were purified from the culture supernatant of the Pol6 strain by gel filtration, ion exchange chromatography, and preparative anionic native electrophoresis. These enzymes were eluted as two distinct species from the diethylamino ethanol Sepharose CL6B and anionic native electrophoresis. However, both behaved identically on sodium dodecyl sulfate polyacrylamide gel electrophoresis (MW, 98 kDa), shared the same amino acid composition, carbohydrate content (8%), and kinetic properties. Moreover, they strongly cross-reacted immunologically. They were active on cellobiose and *p*NPG with *Km* values of 1.43 and 0.37 mM, respectively. β-glu 1 and β-glu 2 were competitively inhibited by 1 mM of glucose and 0.03 mM of δ-gluconolactone. They were also significantly inhibited by Hg^{2+} and Cu^{2} at 2 mM. The addition of purified enzymes to the poor β-glucosidase crude extract of *Trichoderma reesei* increased its hydrolytic efficiency on H_3P0_4 swollen cellulose but had no effect with *P. occitanis* crude extract. Besides their hydrolytic activities, β-glu 1 and β-glu 2 were endowed with trans-glycosidase activity at high concentration of glucose.

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Keywords *Penicillium occitanis* · Pol6 mutant · Cellulolytic system · β-Glucosidase isoforms · Purification

Abbreviations

X-glu	5-bromo,4-chloro,3-indolyl,1,4β-glucopyranoside
Tris	trishydroxymethylaminomethane
EDTA	ethylene-diaminetetraacetic acid
p-NP	*p*-nitrophenol
*p*NPG	*p*-nitrophenyl-β-D-glucopyranoside
PMSF	phenylmethylsulfonyl fluoride

Introduction

Cellulose, a major cell-wall constituent in higher plant, is a linear polysaccharide consisting of β-1,4-linked D-glucose residues. The first step in the saccharification of cellulosic materials involves the synergistic action of endo (E.C 3.2.1.4) and exo-β-1,4 glucanases (E.C 3.2.1.91), yielding short cello-olgosaccharides and cellobiose which are cleaved by β-glucosidases (EC 3.2.1.21). By degrading these products to glucose, β-glucosidases contribute to overcoming the inhibition of cellulases by cellulose hydrolysis end-products [1].

β-Glucosidases are widely distributed in the living world and play pivotal roles in many biological processes. In fact, besides their role in the biological conversion of cellulose to glucose in fungi and bacteria, these enzymes are involved in defense against pathogens and activation of phytohormones in plants [2]. They are also employed in food technology for hydrolysis of bitter compounds during juice extraction and liberation of aroma from wine grapes [3]. β-Glucosidases have also found applications in the synthesis of alkyl- and aryl-glycosides from natural polysaccharides or their derivatives and alcohols by reversed hydrolysis or trans-glycosylation [4]. These products have many potential applications in pharmaceutical, cosmetic, and detergent industries [3, 4].

β-glucosidases from several fungi have been purified to homogeneity and characterized biochemically, but little is known about the cellulolytic system of the filamentous fungus *Penicillium occitanis*. This fungus has been shown to possess a high capacity for cellulases and β-glucosidase production [5–7] with high cellulose degradation efficiency [8]. Two cellobiohydrolases [9] and two low molecular weight endoglucanases were purified from this microorganism [10].

The present study describes the production, purification, and some biochemical properties of two β-glucosidase isoforms secreted by Pol6, a hypercellulolytic mutant of *P. occitanis* grown in the presence of cellulose.

Materials and Methods

Culture Conditions

P. occitanis Pol6 was provided by Professor Tiraby, CAYLA Company, Toulouse-France. This strain is a hypercellulolytic mutant selected by Jain et al. [5] after eight rounds of mutagenesis from the CL100 mother strain. It was grown in Mandels medium [11] containing cellulose Avicel (Merck) at 28 °C in a 7 l fermenter (Chemap, Basel, Switzerland). The pH was maintained at 5.5 with $NaNO_3$, which yields an effectively

buffered medium [8]. After 7 days of fed-batch fermentation, the culture supernatant was separated from the mycelium by centrifugation and filtrated through 0.45 μm nitrocellulose membrane. The crude filtrate was used as the source of cellulases.

Chemicals

p-Nitrophenyl-β-D-glucopyranoside (*p*NPG), 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-glu) and cellobiose were purchased from Sigma Chemical (St Louis, MO, USA). Sephadex G25, diethylamino ethanol (DEAE) Sepharose CL6B columns, and Polybuffer 74 were from Pharmacia.

Enzyme and Protein Assays

β-Glucosidase activity was determined by measuring the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), [12]. The incubation mixture comprised 5 mM *p*NPG, 100 mM sodium acetate buffer pH 4.8, and appropriately diluted enzyme solution in a final volume of 500 μl. The reaction was pre-incubated for 2 min at 60 °C, incubated for 6 min at the same temperature, and then stopped by the addition of 600 μl of glycine buffer (0.4 M, pH 10.8). The amount of *p*-nitrophenol (*p*NP) liberated was determined spectrophotometrically by measuring the A_{430} of the solution. β-Glucosidase activity toward cellobiose was determined by assaying the amount of glucose released by the glucose oxidase/peroxidase method [13]. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol product (i.e., *p*-nitrophenol from *p*NPG or glucose from cellobiose) per minute under the conditions of the assay.

Released reducing sugar was estimated by the 3,5-dinitrosalycylic acid method [14]. The protein concentration at each stage of enzyme purification was determined by the method of Bradford [15] with bovine serum albumin as standard. Proteins in the column effluents were monitored by measuring A_{280}.

Enzyme Purification

All purification steps were performed at 4 °C unless otherwise stated. Proteins in crude extract from the *P. occitanis* Pol6 cultures were precipitated by ammonium sulfate (50%). After centrifugation, the pellet was resuspended in buffer A [20 mM Tris–HCl pH 8; 1 mM ethylene-diaminetetraacetic acid (EDTA); 0.1 mM phenylmethylsulfonyl fluoride and 50 mM NaCl] and was first applied on Sephadex G25 column (5×90 cm) which was previously equilibrated with the same buffer. *p*NPGase-active fractions were pooled and dialyzed against the same buffer and applied on DEAE Sepharose CL6B column (1.6× 20 cm). Elution was carried out with a linear gradient of 0 to 400 mM NaCl with a flow rate of 25 ml/h. Fractions showing *p*NPGase activity were analyzed by a native gel electrophoresis and detected with a chromophoric substrate: 5-bromo-4-chloro3-indolyl-β-D-glucopyranoside (X-glu). Fractions having the same pattern were pooled and concentrated in an Amicon filtration unit with PM30 membrane (Amicon, Lexington, MA, USA). The concentrate was then applied on a preparative native gel electrophoresis as described previously by Laemmli and Favre [16] with 5–20% of acrylamide gel but without sodium dodecyl sulfate (SDS). The electrophoresis was carried out at 4 °C in a gel-casting apparatus (Pharmacia) using the TBE buffer (89 mM Tris, 2 mM EDTA and 89 mM boric acid). After electrophoresis, a portion of the gel was stained for β-glucosidase activity, and the other one was stained by the Coomassie brilliant blue. The characterization of β-

glucosidase activity was made at 37 °C using the X-glu as substrate (100 μg/ml in 100 mM sodium acetate buffer pH 4.8). The X-glu, once diffused in the acrylamide gel, will be hydrolyzed only by the protein band corresponding to the β-glucosidase activity which became blue stained. The band corresponding to the β-glucosidase activity was so excised from the polyacrylamide gel, homogenized in 2 ml of 100 mM sodium acetate buffer (pH 4.8), and incubated at 4 °C for 4 h. The supernatant containing the enzyme was then collected after a centrifugation at 10,000×*g* for 30 min.

Chromatofocusing Method

Pooled fractions containing β-glucosidase activity from the DEAE Sepharose CL 6B were dialyzed against the starting buffer (25 mM histidine-HCl pH 7.4) and applied to the chromatofocusing column (1.6×25 cm) which was previously packed with Polybuffer exchanger PBE 94 TM gel (Pharmacia, France) and equilibrated with the starting buffer. Enzyme elution was carried out by Polybuffer 74, and fractions of 2 ml were collected at a flow rate of 10 ml/h.

Determination of Total Sugar and Amino Acid Composition

The carbohydrate amount of the purified β-glucosidases was determined by the anthrone/H_2SO_4 method using glucose as standard [17].The purified β-glucosidases were hydrolyzed with 6 N HCl and 0.1% of phenol in sealed tube for 24, 48, and 72 h at 110 °C. After removal of the acid excess, the samples were analyzed in amino acid analyzer (Beckman Model 6300).

Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on β-glucosidase activities was determined by incubating the purified enzymes with *p*NPG in 100 mM sodium acetate buffer (pH 4.8) for 6 min at different temperatures ranging from 30 to 70 °C. Thermal stability of the enzymes was determined by assaying for residual enzyme activities after incubation at various temperatures for 6 min without substrate.

Effect of pH on Enzyme Activity and Stability

The optimal pH of β-glucosidase activities was examined at pH 2–9 under standard assay conditions using 50 mM of citrate butter (pH 2 to 6) and Tris–HCl buffer (pH 7 to 9). The effect of pH on β-glucosidase stability was determined using the same buffer system in the range of pH 2–9. The enzyme solution was incubated at various pH values for 24 h at 4 °C without substrate. The remaining enzyme activity was then measured at 60 °C against *p*NPG in 100 mM sodium acetate buffer (pH 4.8).

Kinetic Studies of the Hydrolysis of *p*NPG and Cellobiose by the β-glucosidases

The Michaelis–Menton constant (k_m) and the maximal reaction velocities (V_{max}) were determined using Lineweaver–Burk plots for the β-glucosidase activities by incubating in 100 mM sodium acetate buffer (pH 4.8) at 60 °C with 0.1–1.5 mM *p*NPG or 0.25–1 mM cellobiose. The *p*NP was measured at standard assay conditions. For k_i determination, glucose and δ-gluconolactone concentrations were varied from 0 to 10 mM and 0 to 5 mM, respectively.

Effect of Various Cations

β-Glucosidases were pre-incubated with metal cations in 100 mM sodium acetate buffer (pH 4.8) at 4 °C for 30 min. The residual activity was then measured under the standard assay conditions.

Preparation of the β-Glucosidase Antisera and Immunoblotting

Specific antisera against the purified β-glucosidases were prepared in rabbit as follows: 20 μg of each purified enzyme were emulsified with the Freund's complete adjuvant and injected three times at 12-day intervals. The final immunization was performed 10 days later without the Freund's adjuvant. Rabbit sera were then precipitated with ammonium sulfate and dialyzed against 20 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl. The purified proteins, β-glu l and β-glu 2, were separated via native polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane BA85 (Schleicher and Schuell). Each enzyme was detected using the prepared antisera (1 μg/ml). Anti-rabbit IgG coupled to alkaline phosphatase were used as the second antibodies.

Characterization of Trans-glycosidase Synthesis Products

Five micrograms of purified β-glucosidases and 100 mg of anhydrous glucose were incubated in 100 μl of acetate buffer 100 mM pH 4.8 for 4 h at 60 °C. After centrifugation, most of salts and proteins were removed using ion exchange resin and a SepPack cartridge C18. The supernatant was freeze-dried and dissolved in 50 μl of water. Analysis of sugars was achieved by high-performance liquid chromatography (HPLC) using the BioRad HPX-42A carbohydrate column (300×7.8 mm) and water at 85 °C as eluent with a flow rate of 0.6 ml/min. The column effluent was monitored using a refractive index detector.

Results and Discussion

β-Glucosidase Purification

Isolation of β-glucosidases from *P. occitanis* was carried out using different types of chromatography. During the purification procedures, β-glucosidase activity toward *p*NPG was monotired (Table 1). The first step of purification included ammonium sulfate fractionation followed by gel filtration on Sephadex G25 column to remove pigments and salts. Active protein fractions toward *p*NPG were pooled and applied to a DEAE Sepharose CL 6B column. Protein elution profile is shown in Fig. 1. Several major peaks can be seen on a chromatogram. The fraction eluted with 120 mM NaCl, corresponding to the protein peak marked with an arrow, contained the highest activity against *p*NPG. All fractions of this peak were analyzed directly on polyacrylamide gel electrophoresis under native conditions and stained with the chromophoric substrate X-glu. Figure 2 revealed two bands designated as β-glu1 and β-glu 2 which migrated differently in native polyacrylamide gel. They were eluted from the column of DEAE Sepharose at the beginning (fractions 21–28) and the end (fractions 32–39) of the peak, respectively. Purification of the two proteins was then achieved by preparative nondenaturing polyacrylamide gel electrophoresis (PAGE). After electrophoresis, a part of the gel was incubated with the X-glu substrate. Individual β-glucosidase enzymes were excised from the unstained part of the gel and eluted separately in 20 mM Tris–HCl 100 mM NaCl pH 7 buffer.

Table 1 Purification of β-glucosidases from *P. occitanis*.

Purification step	Specific *p*NPGase activity (U/mg)
Crude extract	23
Sulfate ammonium precipitation	25.1
Sephadex G25	40.5
Anion exchange	153.5
Preparative electrophoresis of β-glu 1	102.5
Preparative electrophoresis of β-glu 2	173

Specific β-glucosidase activities against the *p*NPG substrate was measured at 60 °C in 100 mM sodium acetate buffer pH 4.8.

As it was shown in Table 1, the specific *p*NPGase activities of the purified β-glu1 and β-glu 2 were 102.5 and 173 U/mg, respectively. Such values were found with β-glucosidases of *Penicillium funiculosum* [18], but they were higher than those of *Trichoderma reesei* [19] and *Ruminococcus albus* [20].

Characterization of the β-Glucosidase Enzymes

An apparent molecular mass of 98 kDa was determined for the purified β-glucosidases by SDS-PAGE (Fig. 3). Fungal β-glucosidase molecular masses were previously reported to be between 48 and 313 kDa [21]. The two purified β-glucosidases have close isoelectric points as determined by chromatofocusing. They were, respectively, 5.5 and 5.3. These enzymes were also characterized as glycoproteins containing 8% of carbohydrate.

Structural Data of the β-Glucosidase Isoforms

As shown in Fig. 4, antisera raised against β-glu 1 and β-glu 2 gave a strong cross-reaction with each enzyme but not with the CBHII, an exo-cellobiohydrolase purified from the same

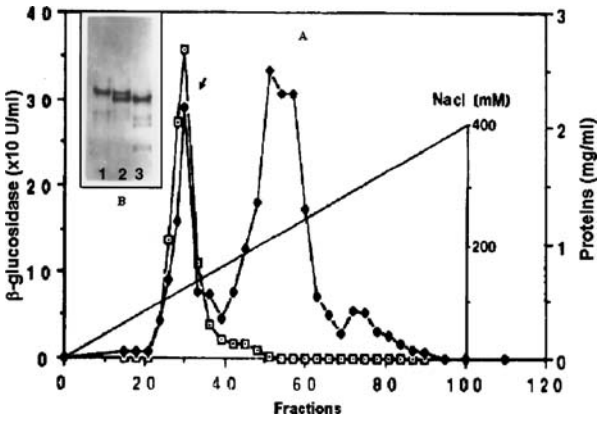

Fig. 1 (*A*) Purification of β-glucosidases using a DEAE Sepharose CL6B. Pooled fractions from the Sephadex G 25 (5×90 cm) column were applied to a DEAE Sepharose CL6B column (20×1.6 cm) previously equilibrated with buffer A: 20 mM Tris–HCl (pH 8), 1 mM EDTA and 50 mM NaCl. The elution was done with a linear gradient of NaCl in the same buffer at a flow rate of 20 ml/h, and fractions of 5 ml were collected. Proteins (*closed squares*), *p*NPG-ase activity (*open squares*). (*B*) Native PAGE analysis of proteins. Native PAGE was performed from fractions number 24 (*lane 1*), number 30 (*lane 2*), and number 35 (*lane 3*)

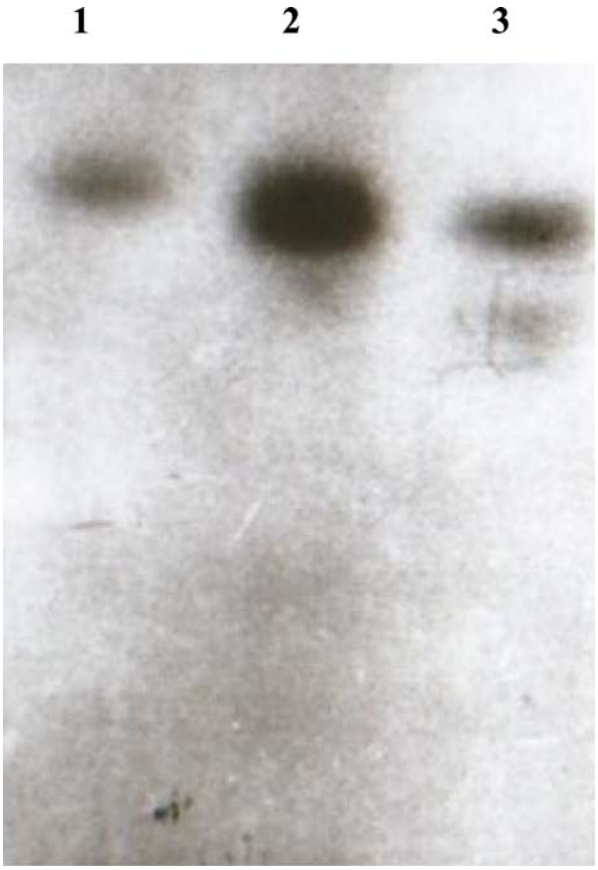

Fig. 2 Electrophoretic characterization of the purified β-glucosidases (β-glu 1 and 2) under non-denaturing conditions and using the X-glu. *Lane 1* β-glu 1 (fraction 24), *lane 2* β-glu 1 and β-glu 2 (fraction 30), *lane 3* β-glu 2 (fraction 35)

fungus [9]. This result suggests that these enzymes share similar primary sequences which is in good agreement with that of β-glu 1 and β-glu 2 exhibiting a very high similarity in their amino acid composition (Table 2).

Temperature Optimum and Heat Stability

The temperature optimum of the purified β-glu 1 and β-glu 2 was determined by performing the standard assay within the temperature range 30–70 °C. Maximum activity was observed at 60 °C for both enzymes. The influence of the temperature on β-glucosidase stability was studied between 30 and 70 °C by pre-incubating the purified enzymes without substrate for 30 min. The residual activity was then assayed with *p*NPG at 60 °C for 6 min. As it was shown in Fig. 5a, the two β-glucosidases maintained 80% of their initial activity after a treatment at 60 °C. However, the stability decreased sharply at 70 °C. This thermostability is comparable to that reported for several β-glucosidases from mesophilic fungi [21].

Effect of pH on Enzyme Activity and Stability

The effect of pH on the activity of the purified β-glucosidases was studied in various buffers over the pH ranges of 2–9. Greatest activity was found to be at pH 4.5, closely

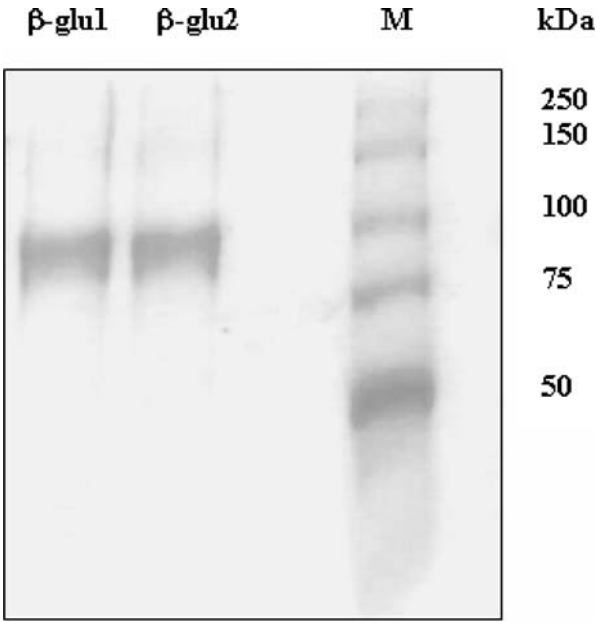

Fig. 3 Analysis of the β-glucosidase purity by SDS-PAGE. Electrophoretical migration was done using a 10% polyacrylamide gel. *Lane 1* 10 μg of β-glu 1; *lane 2* 10 μg of β-glu 2; *lane M* wide-range molecular weight calibration kit

paralleling data reported for other fungi [21]. The stability of β-glu 1and β-glu 2 was examined by pre-incubating them in different pH for 24 h at 4 °C. The remaining activity was then measured against *p*NPG in 100 mM sodium acetate buffer (pH 4.8). The results showed a broad range of stability from pH 3 to 8 for both enzymes (Fig. 5b).

Enzymatic Properties of the β-Glucosidases

The activity of both β-glucosidases was tested on different concentrations of *p*NPG and cellobiose. The initial rates of hydrolysis increased as a function of substrate concentrations to yield a hyperbolic curve (data not shown), as excepted from Michaelis–Menton kinetics. The apparent affinity (Km) and maximum velocitiy (V_{max}) values of β-glu 1 and β-glu 2 activities determined from the Lineweaver–Burk analysis were 0.37 mM and 0.55 μmol min^{-1} for the *p*NPG and 1.43 mM and 0.9 μmol min^{-1} for the cellobiose (Fig. 6). This

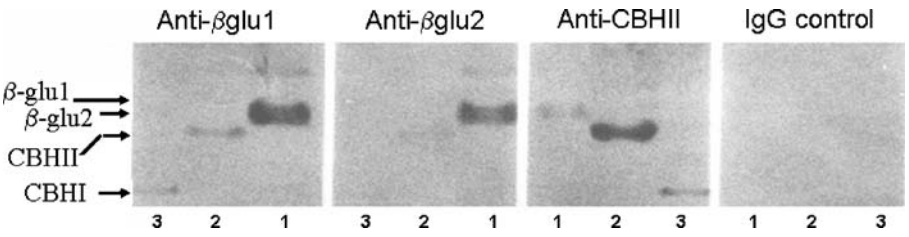

Fig. 4 Immnunoblot of the two β-glucosidases after native electrophoresis. The gel was blotted to the nitrocellulose paper and developed with β-glucosidase antisera as described in "Materials and Methods." *Lane 1* β-glu 1 and β-glu 2 (0.2 μg); *Lane 2* cellobiohydrolase II of *P. occitanis* (0.1 μg); *lane 3* cellobiohydrolase I of *P. occitanis* (0.1 μg)

Table 2 Amino-acid composition of the two β-glucosidases.

Amino acids	Amino acid composition (%)	
	β–glu 1	β-glu 2
Asx	10.0	10.0
Thr	11.8	11.5
Ser	09.1	08.5
Glx	08.8	08.7
Pro	05.2	5.4
Gly	13.1	13.0
Ala	10.1	10.5
Val	07.0	07.5
Met	01.0	01.0
Ile	04.6	04.6
Leu	07.0	07.0
Tyr	03.5	03.5
Phe	02.4	02.5
His	01.1	01.1
Lys	03.0	02.8
Arg	02.9	02.9
Cyc	N.D.	N.D.
Trp	N.D.	N.D.

The purified β-glucosidases were hydrolyzed with 6 N HCl and 0.1% of phenol in sealed tube for 24, 48, and 72 h at 110 °C. The samples were then analyzed in amino acid analyzer.

Asx Asp or Asn; *Glx* Glu or Gln; *N.D* not determined

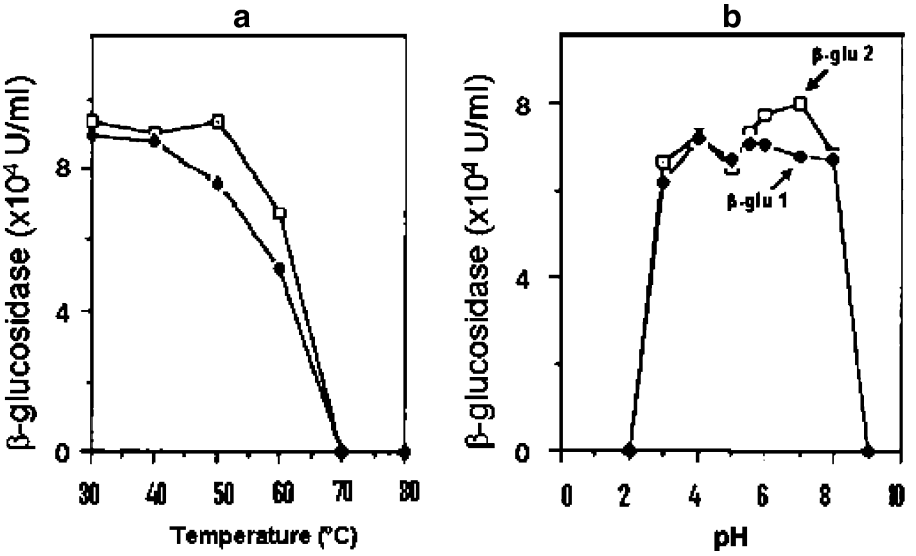

Fig. 5 Effect of temperature and pH on β-glucosidase stability. Thermal stability of the enzymes was determined by assaying for residual enzyme activities after incubation at various temperatures for 6 min without substrate **a**. The enzyme solution was incubated at various pH values for 24 h at 4 °C without substrate. The remaining enzyme activity was then measured at 60 °C against *p*NPG in 100 mM sodium acetate buffer pH 4.8; **b**. *Closed squares* β-glu1; *open squares* β-glu2

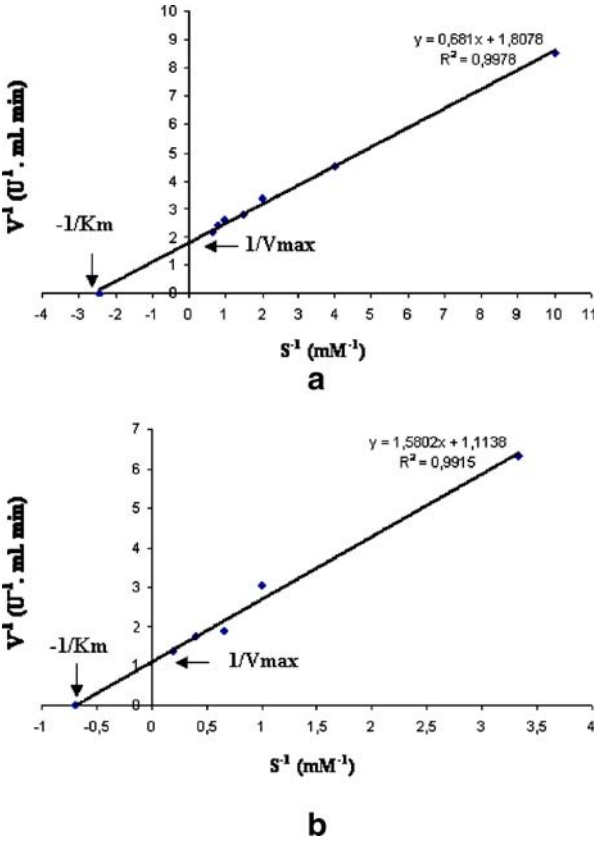

Fig. 6 Lineweaver–Burk analysis of *p*NPG **a** and cellobiose hydrolysis **b**. β-Glucosidases were incubated in 100 mM sodium acetate buffer (pH 4.8) at 60 °C with 0.1–1.5 mM *p*NPG or 0.25–1 mM cellobiose. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol *p*-nitrophenol from *p*NPG or glucose from cellobiose per minute under the conditions of the assay

suggests that β-glu 1 and β-glu 2 are mixed enzymes having aryl-β-glucosidase and cellobiase activities. On the contrary, β-glucosidases from *T. reesei* [22], *Talaromyces emersonii* [23], *Molinia* sp. [24], *Penicillium purpurogenum* [25] and *Aspergillus fumigatus* [26] are aryl-β-glucosidases. The Lineweaver–Burk profiles observed for glucose and δ-gluconolactone exhibited competitive inhibition (Fig. 7). Apparent (K_i) values were equal for both β-glucosidases and were estimated to be 1 and 0.03 mM for glucose and δ-gluconolactone, respectively.

Effect of Bivalent Cations on β-glucosidase Activities

The effect of several divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Cu^{2+}) on the activity of the two enzymes was also studied by adding each ion at a range of concentrations (0.2–5 mM) along with the control. No significant effect on the two enzymes was observed with all the tested cations, except for Hg^{2+} and Cu^{2+} which drastically inhibited the activity at 2 mM (Table 3).

β-Glucosidase Activity on H_3PO_4-Swollen Cellulose

Cellulolytic enzymes are known to act in a synergistic manner to facilitate cleavage of β-1,4-glycosidic bonds of the cellulose. β-Glucosidases play an important role in the

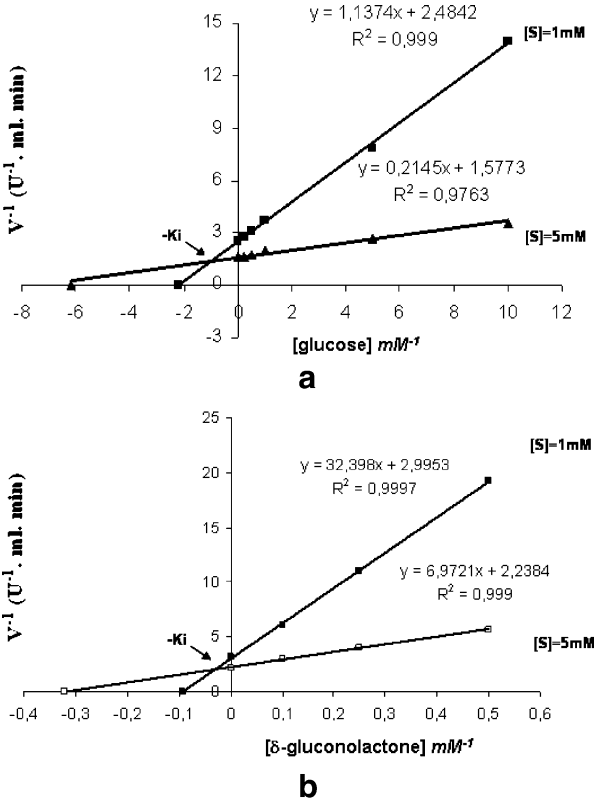

Fig. 7 Lineweaver–Burk plot for the inhibition of β-glu1 or β-glu2 by glucose **a** and δ-gluconolactone **b**. For k_i determination, glucose and δ-gluconolactone concentrations were varied from 0 to 10 mM and 0 to 5 mM, respectively. All the assays were done only with 1 and 5 mM of *p*NPG substrate

complete hydrolysis by converting accumulated cellobiose, a strong inhibitor of other cellulases, to glucose [1].

The influence of the addition of *P. occitanis* β-glucosidases to crude extracts on the cellulose hydrolysis was studied. Crude extracts from *T. reesei* (*Rut* C30 strain) and *P. occitanis* (Pol 6 mutant) were used. The results showed that β-glucosidases supplementation increased the rate of cellulose hydrolysis only when *T. reesei* crude extract is used (Fig. 8). This could be explained by the fact that *T. reesei* cellulolytic complex is poor in β-glucosidases compared to *P. occitanis* ones [7]. Inhibition exerted by cellobiose on *T. reesei*

Table 3 Effect of bivalent cations at 2 mM on β-glucosidase activities.

Bivalent cations	Relative activity (%)	
	β-glu 1	β-glu 2
Hg^{2+}	7	10.9
Cu^{2+}	28	24

β-Glucosidases were pre-incubated with metal cations in 100 mM sodium acetate buffer (pH 4.8) at 4 °C for 30 min. The residual activity was then measured under the standard assay conditions.

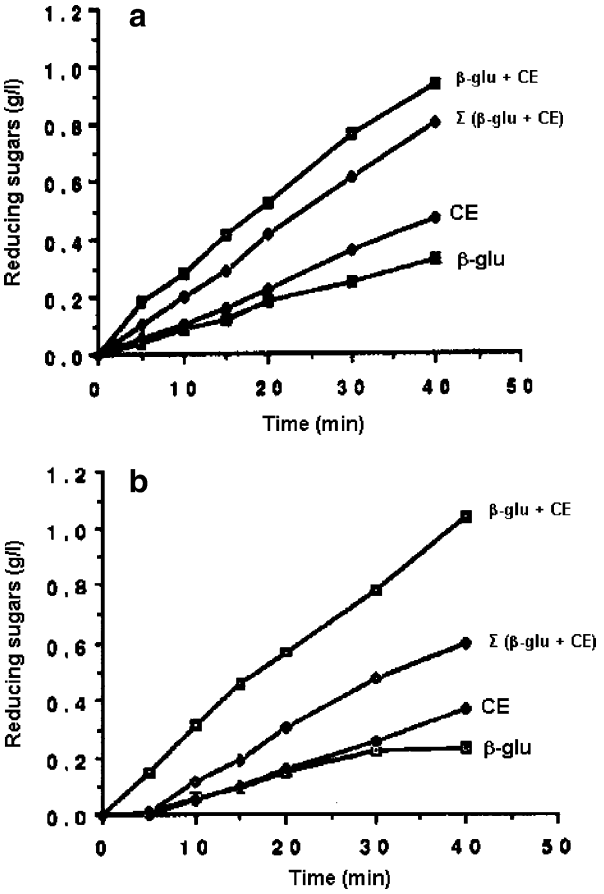

Fig. 8 Effect of β-glucosidase supplementation on H_3PO_4-swollen cellulose. β-glucosidases were added to a *P. occitanis* **a** or *T. reesei* crude extract CE; **b**, either alone or in combination. Mixture was incubated with 1% (*w*/*v*) of substrate for 30 min at 60 °C in 100 °mM sodium acetate buffer (pH 4.8). The amounts of reducing sugars liberated from cellulose were then determined. The *x-axis* indicates the reaction periods. The theoretical activity indicates the sum of individual activities of β-glucosidases and the crude extract (∑ β-glu+CE). The synergistic sum corresponds to the activity of the β-glucosidases and the crude extract mixtures (β-glu+CE)

cellulases is more acute and was overcome by exogenous β-glucosidases enhancing the efficiency of cellulose breakdown.

Trans-glycosidase Activity of the β-Glucosidase Isoforms

Besides their hydrolytic activity, the β-glu 1 and β-glu 2 were also able to carry out trans-glycosylation reactions. In fact, 5 μg of these enzymes was incubated with 1 g/ml of glucose in acetate buffer 100 mM (pH 4.8) at 60 °C. After 4 h, cellobiose formation was detected by HPLC analysis using the BioRad HPX-42A carbohydrate column and water at 85 °C as eluent with a flow rate of 0.6 ml/min (Fig. 9). Trans-glycosidase activity has been also noted with β-glucosidases from mammals [27], plants [28], fungi [29], and bacteria [30].

Conclusion

Tow forms of β-Glucosidase (β-glu 1 and β-glu 2) with molecular mass of 98 kDa were purified to homogeneity from the culturing medium of a hypercellulolytic strain of *P.*

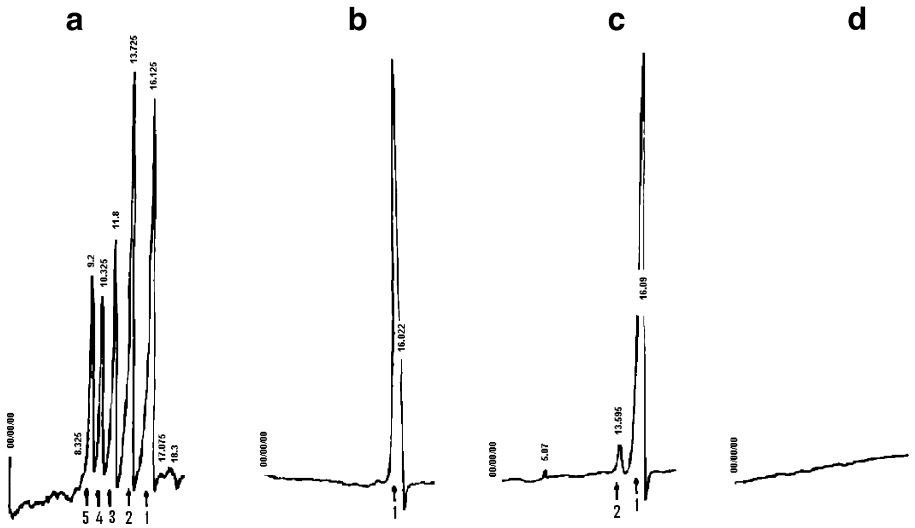

Fig. 9 Trans-glycosidase activity of the *P. occitanis* β-glucosidases. Approximately 100 mg of glucose in 100 mM sodium acetate buffer (pH 4.8) was incubated at 60 °C for 4 h in a total volume of 100 μl, in the absence of enzyme **b**, or in the presence of 5 μg of β-glu 1 or β-glu 2 **c**. Enzyme without substrate was used as control **d**. Standard sugars were used **a**: *peak 1* glucose; *peak 2* cellobiose; *peak 3* cellotriose; *peak 4* cellotetraose, and *peak 5* cellopentaose. All samples were treated under the same conditions, and analysis of sugars was achieved by HPLC using the BioRad HPX-42A carbohydrate column (300×7.8 mm) and water at 85 °C as eluent. The column effluent was monitored using a refractive index detector

occitanis. Their basic characteristics were established. They were secreted as glycoproteins having similar catalytic properties, amino acid composition, and sugar content but differing in their migration by native PAGE. This could be due to post-translational modifications such as phosphorylation which renders β-glu 1 and β-glu 2 charged differently. The possibility that these enzymes have different quaternary structures can be ruled out since they exhibited the same apparent molecular weight as estimated by gel filtration on Biogel P100 (Data not shown). The β-glucosidase polymorphism was also found in other cellulolytic fungi like *Aspergillus niger* [31] and *P. purpurogenum* [25] and was speculated to be associated to the differences in *N*-linked carbohydrate composition. Based on these data, an assumption was made that the purified enzymes represent two forms of β-glucosidase encoded by a single gene. Unfortunately, the intact proteins did not result in any amino acid sequence after Edman degradation due to the blocked N terminus. For this reason, isolation and sequencing of the corresponding gene(s) is needed to confirm this assumption.

Several β-glucosidases have been shown to possess trans-glycosylation activity, and research into their use in oligosaccharide synthesis is being carried out [3, 4]. *P. occitanis* β-glucosidases also showed clear trans-glycosylation activity in high concentration of glucose. Nevertheless, different parameters like substrate concentration, solvent addition, and enzyme immobilization have to be studied in order to optimize the synthesis reactions.

Besides being highly produced by *P. occitanis*, β-glucosidases were very active on cellulose especially when *T. reesei* cellulolytic complex is used. This could be of importance for saccharification of cellulosic materials.

Acknowledgements The authors thank Prof. G. Tiraby and Dr. H. Durand (Cayla Company, France) for kindly supplying the *P. occitanis* strain used in this work. Prof. R. Ellouz is thanked for his continual support and his interest for the subject.

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