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Production, Purification, and Biochemical Characterization of Two β-Glucosidases From *Sclerotinia sclerotiorum*

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Abstract

The filamentous fungus *Sclerotinia sclerotiorum* produces β-glucosidases in liquid culture with a variety of carbon sources, including cellulose (filter paper), xylan, barley straw, oat meal, and xylose. Analysis by native polyacrylamide gel electrophoresis (PAGE) followed by an activity staining with the specific chromogenic substrate, 5-bromo 4-chloro 3-indolyl β-1,4 glucoside (X-glu) showed that two extracellular β-glucosidases, designated as β-glu1 and β-glu2, were in the filter paper culture filtrate. Only one enzyme designated as β-glu x was revealed by the same method in the xylose culture filtrate. β-glu1 and β-glu2 were purified to homogeneity. The purification procedure consist of a common step of anion-exchange chromatography on DEAE–Sepharose CL6B, both high-performance liquid chromatography (HPLC) anion-exchange and gel filtration columns for β-glu1 and only HPLC gel filtration for β-glu2. β-glu1 has a molecular mass of 196 kDa and 96.5 kDa, as estimated by gel filtration and sodium dodecyl sulfate (SDS)–PAGE, respectively, suggesting that the native enzyme may consist of two identical subunits. The same analysis showed that β-glu2 is a monomeric protein with

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an apparent molecular mass of about 76.5 kDa. β-glu1 and β-glu2 hydrolyses PNPGlc and cellobiose, with apparent K_m values respectively for PNPGlc and cellobiose of 0.1 and 1.9 mM for β-glu1 and 2.8 and 8 mM for β-glu2. Both enzymes exhibit the same temperature and pH optima for PNPGlc hydrolysis (60°C and pH 5.0). β-glu1 was stable over a pH range of 3–8 and kept 50% of its activity after 30 min of heating at 60°C without substrate. It was further characterized by studying the effect of some cations and various reagents on its activity.

Index Entries: β-Glucosidase; induction; purification; *Sclerotinia sclerotiorum*.

Introduction

The filamentous fungus *Sclerotinia sclerotiorum* is an important plant pathogen. It possesses a wide range of hosts and causes many diseases, so that consistent control is generally difficult *(1)*. The association of excreted hydrolytic enzymes during the pathogenesis phenomenon (cell wall degradation) has been pointed out *(2,3)*. After secretion, these enzymes remain stable and are active outside the fungal cell *(3)*. The ability of *S. sclerotiorum* to degrade cell walls was observed and it appears that it secretes a battery of hydrolytic enzymes and has a complete cellulolytic system *(2,4)*.

β-Glucosidases (E.C. 3.2.1.21) are widely distributed hydrolases, isolated from a large number of micro-organisms. They are components of cellulase enzymes and have synergetic action with endoglucanases (E.C. 3.2.1.14) and exoglucanases (E.C. 3.2.1.91). Generally, fungi are known to excrete different β-glucosidases (isoenzymes) using a variety of carbon sources as inducers *(5–8)*. These enzymes exhibit variability with respect to substrate specificity and biochemical and kinetic properties.

β-Glucosidases are of interest because of their widely recognized role in cellulose degradation and saccharification of low-residue materials *(9,10)*, their overall importance in the regulation of cellulolysis, and their implication in many biotechnological processes such as synthesis of oligosaccharides, alkyl-glucosides *(11–13)*, or the aromatization of wines and fruit juices *(14,15)*.

To our knowledge, only one β-glucosidase produced by *Sclerotinia sclerotiorum* on carboxymethylcellulose (CMC) culture filtrate has previously been described *(16)*. In the present article, we describe the production, purification, and partial characterization of two novel β-glucosidases (β-glu1 and β-glu2) produced by *S. sclerotiorum* when grown on filter paper as the carbon source.

Materials and Methods

Organism and Culture Conditions

Sclerotinia sclerotiorum was isolated from the sunflower. It was obtained from the Laboratoire de Cryptogamie INRAT—TUNISIE. The fungus was maintained on potato dextrose agar. For enzyme production,

S. sclerotiorum was grown on minimal medium [1 g/L KCl, 0.5 g/L $MgSO_4$, 1 g/L KH_2PO_4, 4.3 g/L $NaNO_3$, 1.4 g/L $(NH_4)_2SO_4$, 2 g/L yeast extract, 0.5% potassium phtalate, pH 5.5, and 1 mL/L of an oligo-element solution containing 2 g/L $CoCl_2$, 1.6 g/L $MnSO_4H_2O$, 1.4 g/L $ZnSO_4H_2O$, 5 g/L $FeSO_47H_2O$] supplemented with an appropriate inducer (filter paper, xylan, barley straw, oat meal, xylose, or glucose) at a concentration of 1% (w/v). The cultures were incubated at 25°C for 12 d on an orbital shaker (0.4*g*) and were harvested by centrifugation and filtration through Whatman GF/A glass microfiber filters. The filtrates were used as the crude enzyme preparations.

Enzyme Assay

β-Glucosidase activity was measured using *para*-nitro-phenyl-β-D-glucopyranoside (PNPGlc) as the substrate. The enzyme was incubated at 60°C with 0.5 m*M* of PNPGlc solution in 50 m*M* acetate buffer (0.5 mL) at pH 5 for 30 min. The reaction was stopped by adding 0.6 mL of a 0.4 *M* glycine–NaOH buffer (pH 10.8) and the released *p*-nitrophenol was monitored by measuring the absorbance at 430 nm (A_{430}). The extinction coefficient used was 18,300 mol^{-1} cm^{-1}. Enzyme activity for the characterization of β-glu1 and β-glu2 in purified preparations was assayed using the same procedure except that the reaction was terminated after 5 min incubation. One unit of β-glucosidase activity corresponds to the release of 1 µmol of *para*-nitro phenol per minute under these conditions.

The hydrolysis of cellobiose to glucose (cellobiase assay) was carried out using a cellobiose concentration of 5 m*M*. The released glucose was estimated with oxidase/peroxidase reagent (glucose enzymatic color liquid kit; Merck). One unit of β-glucosidase activity corresponds to the release of 1 µmol glucose/min at pH 5.0 and 60°C for 30 min.

Purification Procedure

Purification steps were carried out at 4°C.

Common Step of DEAE–Sepharose CL6B Column Chromatography

The pH of the crude enzyme preparation was adjusted to pH 7.25 and loaded onto a DEAE–Sepharose CL6B column (15 × 3 cm) pre-equilibrated with 25 m*M* Tris-HCl buffer (pH 7.25). The column was washed extensively with the same buffer and eluted with a gradient of 0–0.5 *M* NaCl (200 mL) at a flow rate of 50 mL/h; 2.5-mL fractions were collected.

The first half of the active fractions were pooled, desalted, and concentrated by ultrafiltration (Amicon PM 10 membrane) and used for the purification of β-glu1, whereas the other half of the fractions served for the purification of β-glu2.

Purification of β-Glu1

The β-glu1 was further purified by high-performance liquid chromatography (HPLC) on an anion-exchange column (TSK DEAE–5PW, 75 ×

7.5 mm) equilibrated with 25 m*M* Tris-HCl buffer (pH 7.25). The elution was carried out by a 500-m*M* NaCl linear gradient in the same buffer at a flow rate of 0.5 mL/min. Proteins were monitored by determining the absorbance at 280 nm (A_{280}). The β-glucosidase activity was eluted as a single peak. Peak fractions concentrated as earlier were injected onto a HPLC gel filtration column TSK 3000 SW (60 cm × 7.5 mm) pre-equilibrated with 50 m*M* Tris-HCl buffer (pH 7.0) with a flow rate of 0.8 mL/min. The β-glucosidase activity was eluted as a single peak that was recovered in four fractions of 0.5 mL each. Reinjection of the second fraction allowed finding a single protein peak. This fraction corresponded to the purified β-glu1 preparation and served for the subsequent studies.

Purification of β-Glu2

Fractions from DEAE–Sepharose CL6B column with β-glu2 was desalted and concentrated by ultrafiltration on a centriplus PM 10 membrane (Amicon). The resulting sample was injected onto a 60-cm × 7.5-mm TSK gel 3000 SW column, at a flow of 0.5 mL/min with 100 m*M* NaCl and 50 m*M* phosphate buffer (pH 7.0). Proteins were monitored by A_{280}. Two β-glucosidase peaks were obtained. Identification of the corresponding enzymes was carried out after determination of molecular mass using standards proteins and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Thus, the second peak corresponded to the purified β-glu2 preparation, its purity was confirmed by both SDS-PAGE and reinjection on a TSK gel 2000 SW HPLC gel filtration column, with the same buffer and under the same conditions, as cited earlier.

Determination of Molecular Mass

The molecular mass of native proteins was determined by HPLC gel filtration on a TSK gel 3000 SW column (60 cm × 7.5 mm). The molecular mass of the purified β-glucosidases was estimated with standard markers (Bio-rad): thyroglobin, 670 kDa; bovine γ-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B_{12}, 1.35 kDa.

Protein Determination

The protein concentration was determined by the method of Bradford *(17)* with bovine serum albumin (BSA) as the standard.

Polyacrylamide Gel Electrophoresis

The purity of the enzyme preparations was determined by SDS-PAGE performed with a 10% polyacrylamide gel according to the Laemmli method *(18)* by using a Dual SLAB gel unit, Model DSG-125 system. The gel was stained with silver nitrate *(19)*. The molecular mass of the subunits was estimated with standard markers (HMW, Sigma). Native PAGE was performed at 4°C with a 10% polyacrylamide gel *(18)*. β-Glucosidases were visualized by staining the gel with 50 m*M* acetate buffer (pH 5.0) containing

40 µg/mL of the specific chromogenic substrate, X-glu (5-bromo 4-chloro 3-indolyl β-1,4 glucoside) and incubation at 50°C for several minutes until blue bands appeared.

Kinetic Properties

The Michaelis-Menten constant (K_m) and the maximal reaction velocities (V_{max}) were determined for β-glu1 and β-glu2 by incubating them at pH 5.0 and 60°C with PNPGlclc or cellobiose in concentrations ranging from 0.05 to 0.5 m*M* PNPGlc and from 0.25 to 10 m*M* cellobiose respectively. The reactions were stopped after 5 min and the *para*-nitro phenol (pNP) or glucose that was produced was measured at standard assays conditions. All assays were performed in duplicate. Values for K_m and V_{max} were determined from Lineweaver–Burk plots.

Effect of pH on β-*Glucosidase Activity and Stability*

The optimum pH of activity was determined by monitoring each activity at 60°C at various pH values ranging between 2.0 and 9.0. The following buffers were used: 50 m*M* glycine-HCl (pH 2.0 and 3.0); 50 m*M* acetate (pH 4.0–6.0); Tris-HCl (pH 7.0–9.0). The β-glucosidase stability was examined at the pH values 2.0–9.0. Enzyme samples were preincubated in the above-cited buffers at 4°C for 24 h before adding the substrate. After adjustment of pH, the residual activity was determined under standard conditions by the PNPGlc assay.

Effect of Temperature on β-*Glucosidase Activity and Stability*

The optimum temperature for enzyme activity was determined by monitoring each activity at pH 5.0 at various temperatures (4–80°C). Stability against heat inactivation was studied by preincubation of enzyme samples for 30 min at various temperatures in acetate buffer (50 m*M*; pH 5.0) and then determining under standard conditions the residual enzymes activities (PNPGlc assay).

Results and Discussion

Production of β-*Glucosidases*

The effect of a variety of carbon sources on β-glucosidase production by *S. sclerotiorum* in shake flasks is presented in Fig. 1. The results show that a β-glucosidase activity can be induced by carbon sources such as filter paper (cellulose), xylan, oat meal, barley straw, and xylose. β-Glucosidase production was repressed when using glucose as a carbon source. Screening of β-glucosidases from filter paper and xylose culture filtrates by native PAGE and activity stain allowed us to find differential induction. Two different β-glucosidase activity bands were found on filter paper culture filtrate (*see* Fig. 2). They were designated respectively β-glu1 and β-glu2. On xylose culture filtrate, another β-glucosidase activity band, different from the two preceding ones, was found; it was designated β-glu x (*see* Fig. 2).

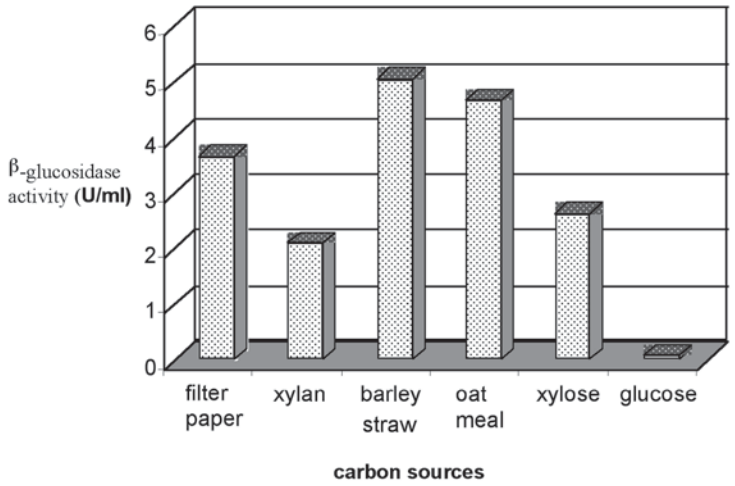

Fig. 1. Effect of carbon source on β-glucosidase production by *S. sclerotiorum*. The fungus was grown in liquid culture with 1% (w/v) inducers at 25°C for 12 d.

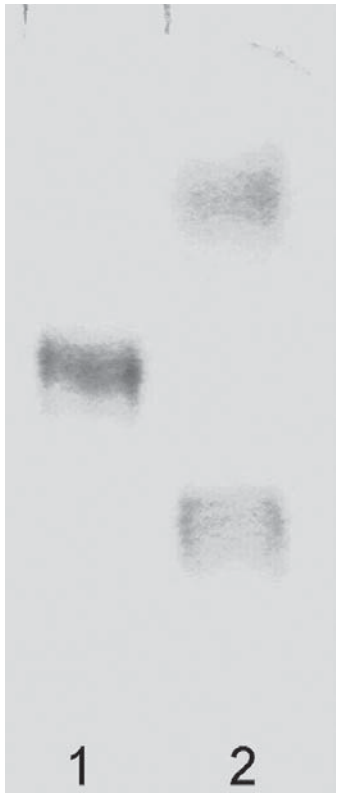

Fig. 2. Native PAGE of crude extracts from *S. sclerotiorum* produced with xylose (lane 1) and filter paper (lane 2) as inducers. The polyacrylamide gel (10%) was stained with the chromogenic substrate X-glu (40 µg/mL) in 50 m*M* acetate buffer (pH 5.0) at 50°C.

The ability of the fungus to grow on lignocellulosic residues (barley straw and oat meal) with high production of β-glucosidase activity and other polysaccharide hydrolases (data not shown) represents a great advantage for the saccharification and conversion of these low-grade residues, especially for bioethanol production. Several articles have described such application *(9,10,20,21)*. These results indicate that *S. sclerotiorum* possesses a complete battery of hydrolytic enzymes (cellulases, xylanases, pectinases, etc.) and seems to possess efficient cellulolytic and xylanolytic systems *(22,23)*. In a previous work, Waksman *(16)* has described the production and the purification from *S. sclerotiorum* of one β-glucosidase activity in CMC culture filtrate. It, however, exhibits different properties. We note here that the origin of the fungus is not the same. Furthermore, the existence of differential induction of β-glucosidase enzymes as we have shown by native PAGE may explain this result. Several fungi were found to produce β-glucosidases on different carbohydrates as carbon sources *(24–27)*, and multiple forms of β-glucosidase have been found in the culture broth of a variety of microbes *(28–31)*.

Purification and Molecular Mass Determination

Table 1 summarizes the results of the purification procedures of β-glu1 and β-glu2 from the culture supernatant of *S. sclerotiorum* cultivated with paper filter as the carbon source. Conditions and protocols of purification are indicated in the Materials and Methods section.

Both β-glu1 and β-glu2 were purified from a common step, consisting of a DEAE–Sepharose anion-exchange chromatography (*see* Fig. 3). The β-glucosidase activity was eluted as a large active peak with a clear shoulder showing the two enzymes β-glu1 and β-glu2. No activity was observed in the column filtrate. β-glu1 was further purified by HPLC using an anion-exchange column followed by a gel filtration column. The purity of the β-glu1 preparation was checked by analytic HPLC gel filtration and SDS-PAGE (*see* Fig. 4). The molecular mass of the purified β-glu1 was determined to be 96.5 and 196 kDa, as estimated by SDS-PAGE and gel filtration, respectively (*see* Fig. 4). These results suggest that the purified β-glu1 from *S. sclerotiorum* is composed of two identical subunits. Thus, the native enzyme is probably an homodimer.

The β-glu2 was only purified by a single HPLC gel filtration step (*see* Table 1). We have omitted the HPLC anion-exchange step. In fact, it did not give any recovery improvement in the purification procedure of β-glu1. However, we have improved the separation using the TSK 3000 HPLC gel filtration column by lowering the flow rate (0.5 mL/min) and changing the mobile phase, to 50 m*M* phosphate buffer, pH 7.0, containing 100 m*M* NaCl. Results are reported in Fig. 5. Two β-glucosidase peaks were found (peak I and peak II), they were subsequently identified as β-glu1 (peak I) and β-glu2 (peak II). Thus, β-glu2 appears to have a native molecular mass of 79 kDa. Analytic injection of the fraction corresponding to the top of peak II in an another gel filtration column (TSK 2000) allowed to verify the

Table 1
Summary of β-glu1 and β-glu2 Enzyme Purification From Filter Paper (Cellulose) Culture Filtrate of *S. sclerotiorum*

	Specific activity (U/mg)		Purification (fold)	
Purification step	β-glu1	β-glu2	β-glu1	β-glu2
Culture filtrate	7.6		1	
DEAE–Sepharose CL6B	180	158	23.5	21
TSK–DEAE–5PW	190	—	25	—
TSK 3000 SW	277.5	184	36	24

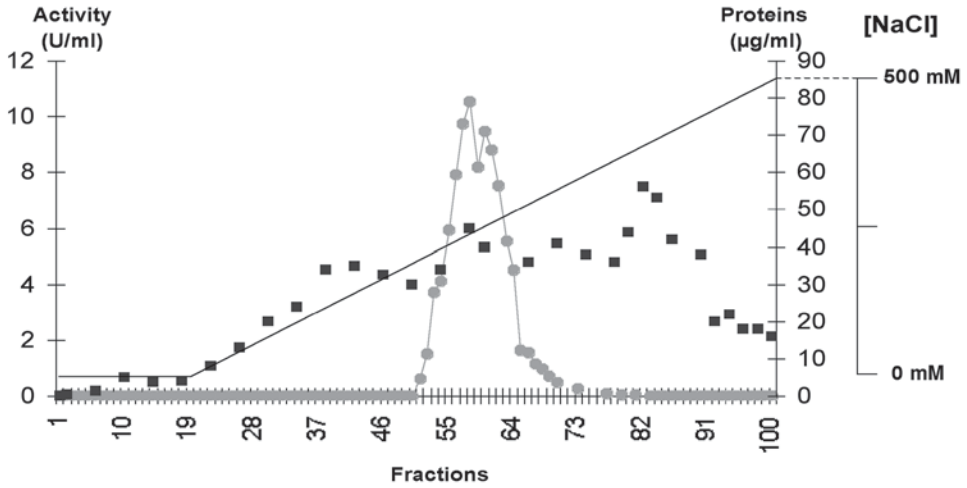

Fig. 3. Elution pattern of β-glucosidase activity from DEAE–Sepharose CL6B column; (●) β-glucosidase activity; (■) proteins; solid line: NaCl gradient.

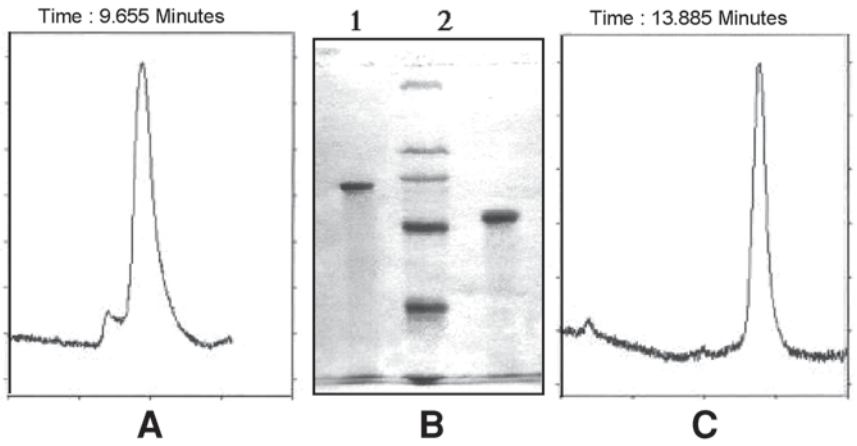

Fig. 4. Final steps of purification of β-glu1 and β-glu2. **(A)** Analysis of purified β-glu1 preparation on TSK 3000 HPLC gel filtration column. **(B)** SDS-PAGE analysis of β-glu1 and β-glu2 in a 10% polyacrylamide gel stained with silver nitrate: lane 1, purified β-glu1; lane 2, molecular-weight markers (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66.3 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa); lane 3, purified β-glu2. **(C)** Analysis of purified β-glu2 preparation on TSK 2000 HPLC gel filtration column.

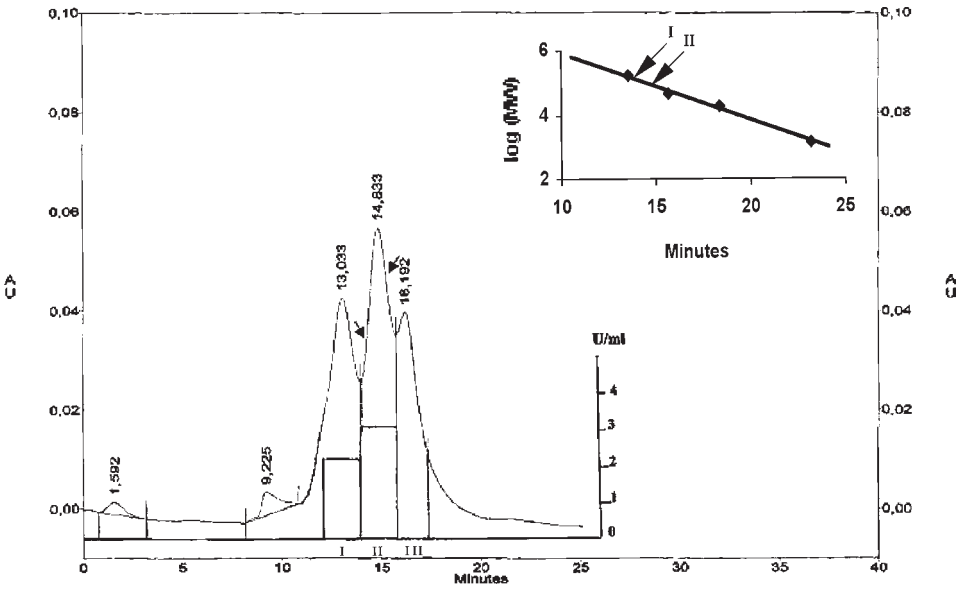

Fig. 5. Purification of β-glu2 with HPLC gel filtration on a TSK 3000 SW column. The column was equilibrated and eluted with 100 m*M* NaCl and 50 m*M* phosphate buffer (pH 7.0) at 0.5 mL/min. Proteins were monitored by determining the absorbance at 280 nm (AU). β-Glucosidase activity is represented by histograms and expressed in U/mL. Molecular mass determination showed that peak I corresponded to the β-glu1 enzyme and peak II to the β-glu2 enzyme.

preparation purity and confirm the β-glu2 molecular mass (*see* Fig. 4). Analysis by SDS-PAGE of the peak II fraction showed one polypeptide of 76.5 kDa (*see* Fig. 4). These results showed that β-glu2 appears to be a monomeric protein.

Determination of molecular mass and number of subunits for β-glu1 and β-glu2 allowed us to compare them to several β-glucosidases from different sources. Similar results were found for other enzymes *(31–35)*. As cited earlier, Waksman *(16)* has described the purification of a β-glucosidase produced by *S. sclerotiorum* in CMC culture filtrate. This enzyme consists of four subunits with a total molecular weight of 260,000. It is possible that the analysis concerned an associated form of the two enzymes, β-glu1 and β-glu2. We have showed, either by native PAGE or by chromatography, fractionation that the two forms β-glu1 and β-glu2 are really separated.

Biochemical Properties

Table 2 summarizes some properties of β-glu1 and β-glu2. The apparent Michaelis–Menten constant (K_m) and the maximal reaction velocities (V_{max}) of the two enzymes with PNPGlc and cellobiose as substrates are also listed in Table 2. Values showed that β-glu1 and β-glu2 have high affinity to PNPGlc and cellobiose. In this respect, β-glu1 and β-glu2 resemble many

Table 2
Some Properties of β-glu1 and β-glu2 From *S. sclerotiorum*

	β-glu1	β-glu2
Native molecular weight (MW) (kDa)	196,000	79,000
SDS-PAGE MW (kDa)	96,500	76,500
Optimum pH at 60°C	5.0	4.5–5.0
Optimum temperature (°C) at pH 5	60	60
pH stability	Stable from pH 3.0 to 8.0	—
Temperature stability	Keeps 50% of its activity for 30 min at 60°C	—
K_m (PNPGlc)	0.1 m*M*	1.9 m*M*
K_m (cellobiose)	2.8 m*M*	8 m*M*
V_{max} (PNPGlc)[a]	265 U/mg	190 U/mg
V_{max} (cellobiose)[b]	150 U/mg	95 U/mg

[a]One unit of β-glucosidase activity corresponds to the release of 1 μmol pNP/min at pH 5.0 and 60°C.
[b]One unit of β-glucosidase activity corresponds to the release of 1 μmol glucose/min at pH 5.0 and 60°C.

β-glucosidases of microbial origin *(5,6)*. β-Glucosidases may be divided into three groups on the basis of substrate specificity: (1) aryl β-glucosidase (which hydrolyzes exclusively aryl-β-glucosides), (2) cellobiase (which only hydrolyzes cellobiose and short-chain cellodextrin), and (3) broad-specificity β-glucosidases, which show activity on both substrate types *(27,36)*. β-glu1 and β-glu2 from *S. sclerotiorum* seem to belong to the most common group, with cellobiase and aryl β-glucosidase activities.

The effect of several ions of metals (Co^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}) and other agents (DTT, EDTA, DMSO, and glycerol) on the β-glu1 activity were tested (*see* Table 3). No significant effect on the PNPGlclc hydrolysis activity was observed with DTT, EDTA, glycerol, Co^{2+}, Mg^{2+}, and Ca^{2+} (*see* Table 3). Under the same conditions, the enzyme activity was stimulated about 20% by DMSO and Mn^{2+}. However, β-glu1 was inhibited by, Zn^{2+}, Cu^{2+}, and Fe^{2+} (*see* Table 3). Inhibition by Zn^{2+}, Cu^{2+}, and Fe^{2+} may be the result of complex formation and/or catalysis of oxidation of specific residues (thiol groups) or nonspecific salt formation *(27,36)*. The chelating agent EDTA did not affect β-glu1 activity, indicating that β-glu1 is not a metalloprotein. Comparably, DTT is not an inhibitor, suggesting that disulfide bonds are not essential for the enzyme activity. Activation by Mn^{2+} and DMSO may be explained by the stabilization of the enzyme structure.

In conclusion, we have shown in this article the differential induction of β-glucosidase activities by *S. sclerotiorum* according to inducer type. The fungus showed a great ability to grow on a variety of carbon sources, including lignocellulosic wastes (barley straw and oat meal), which may constitute a novel method of the saccharification and conversion of those substrates. We have purified and partially characterized two β-glucosi-

Table 3
Effects of Some Cations and Other Reagents
on β-glu1 Activity From *S. sclerotiorum*

Cations or reagents[a]	Relative activity (%)[b]
None	100
Co^{2+}	100
Mg^{2+}	102
Ca^{2+}	106
Mn^{2+}	121
Zn^{2+}	57
Cu^{2+}	10
Fe^{2+}	3
Glycerol	99
DMSO	123
DTT	102
EDTA	101

Note: DMSO = dimethyl sulfoxide; DTT = dithiothreitol.

[a]All cations or reagents are tested at the concentration of 5 m*M*, except DMSO, which was tested at 2 m*M*.

[b]The relative activity was determined by measuring the β-glu1 activity at 60°C and pH 5.0 after preincubation at 4°C for 30 min in the presence of an individual cation or reagent. The activity assayed in the absence of cation or reagent was taken as 100%.

dases (β-glu1 and β-glu2) produced when the fungus is grown on filter paper (cellulose) as its carbon source. When comparing the determined properties to other fungal β-glucosidases, β-glu1 and β-glu2 may be used in some biotechnological applications. Their use in oligosaccharides and alkyl glucosides synthesis is under investigation.

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