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A β-Glucosidase From *Sclerotinia sclerotiorum*

Biochemical Characterization and Use in Oligosaccharide Synthesis

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Abstract

The filamentous fungus *Sclerotinia sclerotiorum*, grown on a xylose medium, was found to excrete one β-glucosidase (β-glu x). The enzyme was purified to apparent homogeneity by ammonium sulfate precipitation, gel filtration, anion-exchange chromatography, and high-performance liquid chromatography (HPLC) gel filtration chromatography. Its molecular mass was estimated to be 130 kDa by HPLC gel filtration and 60 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis, suggesting that β-glu x may be a homodimer. For *p*-nitrophenyl β-D-glucopyranoside hydrolysis, apparent K_m and V_{max} values were found to be 0.09 m*M* and 193 U/mg, respectively, while optimum temperature and pH were 55–60°C and pH 5.0, respectively. β-Glu x was strongly inhibited by Fe^{2+} and activated about 35% by Ca^{2+}. β-Glu x possesses strong transglucosylation activity in comparison with commercially available β-glucosidases. The production rate of total glucooligosaccharides (GOSs) from 30% cellobiose at 50°C and pH 5.0 for 6 h with 0.6 U/mL of enzyme preparation was 80 g/L. It reached 105 g/L under the same conditions when using cellobiose at 350 g/L (1.023 *M*). Finally, GOS structure was determined by mass spectrometry and ^{13}C nuclear magnetic resonance spectroscopy.

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

Introduction

The filamentous fungus *Sclerotinia sclerotiorum* is an important plant pathogen that possesses a wide host range *(1)*. It is known to be a great producer of a large number of cell wall–degrading enzymes, such as cellulases, xylanases, and pectinases *(1–4)*. Generally, these enzymes are stable and active outside the fungal cell after secretion *(5)*.

β-Glucosidases (EC 3.2.1.21) are a component of cellulase enzymes. They catalyze the hydrolysis of β-glucosidic linkages between glucose and alkyl, aryl, or saccharide groups, and they have a synergetic action on the degradation of cellulose, with endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91). β-Glucosidases have been used in many biotechnological applications that exploit either their hydrolytic properties, such as the aromatization of wines and fruit juices *(6,7)*, the saccharification of cellulolytic low-grade materials *(8,9)*, or their activity in synthesis of alkyl-glucosides and oligosaccharides *(10–12)*.

Oligosaccharides and their derivatives play a key role in many biochemical reactions, and their use as therapeutics, as diagnostic tools, and in the food and cosmetics industries is well established *(10,13–17)*. Chemical synthesis of these compounds is very complex and time-consuming *(18,19)*. By contrast, the desired oligosaccharides can be synthesized using either glycosyl hydrolases or glycosyl transferases. This alternative route is not only very promising and attractive, but also capable of yielding highly stereo- and regioselective products *(10,16)*.

In a previous work, we showed *(20)* a differential induction of β-glucosidase enzymes in *S. sclerotiorum*. Two β-glucosidases, designated β-glu1 and β-glu2, and one enzyme, designated β-glu x, were produced, respectively, on filter paper (cellulose) and xylose, as carbon sources. In this article, we describe the purification and characterization of the β-glu x enzyme and its use in the transglycosylation reaction of cellobiose. The transglycosylated products were purified, and their molecular weights and structures were determined by mass spectrometry (MS) and ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

Materials and Methods

Organism and Culture Conditions

S. sclerotiorum was isolated from sunflower (local isolate from Tunisia). It was obtained from the fungi collection of the Laboratoire de Cryptogamie INRAT–Tunis. For β-glu x production, xylose was used at 1%. Culture conditions, medium composition, and preparation of crude enzyme preparation were as described by Smaali et al. *(20)*.

Enzyme Assays

β-Glucosidase was tested in a reaction mixture (0.5 mL) containing 0.5 m*M p*-nitrophenyl β-D-glucopyranoside (pNPGlc), 50 m*M* acetate buffer (pH 5.0), and appropriately diluted enzyme solution. After incubation at 60°C for 10 min, the reaction was stopped by adding 0.6 mL of a 0.4 *M* glycine-NaOH buffer (pH 11.0), and the *p*-nitrophenol (pNP) released was monitored by determining the absorbance at 400 nm (A_{400}). The extinction coefficient used was 18,300 mol/cm. One unit of β-glucosidase activity corresponded to the release of 1 μmol of pNP/min under these conditions.

β-Xylosidase activity was measured by the pNP liberated from *p*-nitrophenyl β-D-xylopyranoside. Conditions were similar to those described for β-glucosidase except the reaction buffer; it consisted of citrate sodium (100 m*M*) containing 250 m*M* NaCl. One unit of β-xylosidase activity corresponded to the release of 1 μmol of pNP/min under these conditions.

Endoglucanase and xylanase activities were assayed on the basis of reducing sugars cleaved from carboxymethylcellulose (CMC) and xylan, respectively. One percent substrate (CMC or xylan) was incubated with an appropriate enzyme solution at 55°C for 30 min. The concentration of reducing sugars was measured by the dinitrosalicylic acid method *(21)* and expressed as glucose or xylose equivalents. One unit of enzyme activity corresponded to the equivalent glucose or xylose liberated per minute at these conditions for endoglucanase or xylanase, respectively.

Polyacrylamide Gel Electrophoresis

The purity of the enzyme preparations was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed with a 10% polyacrylamide gel according to the Laemmli method *(22)* by using a Dual SLAB gel unit, Model DSG-125 system. The gel was stained with silver nitrate *(23)*. Molecular mass of the subunits was estimated with standard markers (high molecular weight; Sigma, St. Louis, MO).

Native PAGE was performed at 4°C with a 10% polyacrylamide gel *(22)*. β-Glucosidase was visualized by staining the gel with 50 m*M* acetate buffer (pH 5.0) containing 40 μg/mL of the specific chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-1,4-glucoside (X-glu), and incubating at 50°C for several minutes until a blue band appeared.

Purification Procedure

All steps were carried out at 4°C.

Ammonium Sulfate Treatment

The crude extract was treated with ammonium sulfate (80% saturation) and left over night. The precipitate was collected by centrifugation at 18,100*g* for 30 min and dissolved in 50 m*M* Tris-HCl (pH 7.0).

Gel Filtration

The concentrated enzyme solution was loaded onto a Sephadex G150 column (112 × 2.2 cm) equilibrated with 50 m*M* Tris-HCl (pH 7.0). Elution was performed with the same buffer at a flow rate of 10 mL/h, and 5-mL fractions were collected. Proteins were monitored by determining the absorbance at 280 nm. β-Glucosidase, β-xylosidase, endoglucanase, and xylanase activities were tested.

Anion-Exchange Chromatography

Gel filtration fractions that contained β-glucosidase activity were pooled, dialyzed against 25 m*M* Tris-HCl buffer (pH 7.0), and chromatographed on DEAE Sepharose CL6B. The column (9.5 × 1.6 cm) was equilibrated by 50 m*M* Tris-HCl (pH 7.0). Elution was carried out with 200 mL of NaCl gradient in the same buffer, with a flow rate of 25 mL/h. Two-milliliter fractions were collected. Proteins were determined following the method of Bradford *(24)*. β-Glucosidase, β-xylosidase, endoglucanase, and xylanase activities were tested.

HPLC Gel Filtration

Active fractions obtained from the last step were pooled, concentrated by ultrafiltration (Centriplus, Amicon PM 10), and injected on a TSK-GEL G2000SWG column (60 cm × 7.5 mm) (Toyo Soda Manufacturing, Japan) equilibrated and eluted at a flow rate of 0.8 mL/min with phosphate buffer (50 m*M*, pH 7.0) containing 100 m*M* NaCl. Proteins were monitored by determining the absorbance at 280 nm (Beckman HPLC, system Gold, equipped with a Beckman 166 detector). The β-glucosidase activity was recovered as a single peak, which corresponded to the pure β-glu x.

Biochemical Properties

The native molecular mass of β-glu x was determined in the HPLC gel filtration purification step by injection of standard markers (Bio-Rad, Hercules, CA): thyroglobin, 670 kDa; γ-globulin bovine, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B_{12}, 1.35 kDa.

Molecular mass of the subunits was assessed by SDS-PAGE using a 10% gel with standard markers (high molecular weight; Sigma): myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin (BSA), 66.3 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa.

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

The optimum temperature for pNPGlc hydrolysis was determined by monitoring each activity at pH 5.0 at various temperatures (4–80°C). Stability against heat inactivation was determined by assaying (by the pNPGlc assay) the remaining activity after preincubation for 30 min at the appropriate temperatures.

The apparent Michaelis-Menten constant (K_m) and the maximal reaction velocity (V_m) were determined for β-glu x by its incubation at 60°C and pH 5.0 with pNPGlc in concentrations ranging from 0.05 to 0.5 m*M*. The released pNP was measured at standard assay conditions. All assays were performed in duplicate. Values for K_m and V_{max} were determined from a Lineweaver-Burk plot.

Glucooligosaccharide Synthesis and Analysis

Cellobiose was employed as the substrate. Glucooligosaccharide (GOS) synthesis was performed at 50°C in acetate buffer (50 m*M*, pH 5.0) supplemented with cellobiose (300 g/L) and β-glu x (0.6 U/mL). The reaction was magnetically stirred and was stopped by heating for 5 min at 100°C. The oligosaccharides formed were analyzed by HPLC (HP series 1100 equipped with an automatic injector HP series 1050 and a differential refractometer, Waters 410) using glucose, cellobiose, cellotriose, and cellotetraose as standards (Sigma). The column used was a C_{18} (Prontosyl, 5 µm, 250 × 4 mm; ICS France). Elution was performed at 30°C by ultrapure water at a constant flow rate of 0.5 mL/min.

Yields of GOS, glucose, or cellobiose are a weight percentage of the appropriate carbohydrate based on total saccharides in the reaction medium. They were expressed as grams/liter by converting their percentage according to the initial cellobiose (substrate) concentration.

The effect of substrate (cellobiose) concentration was studied. It was increased from 0 to 35% (0 to 350 g/L) using the following values: 0, 5, 10, 15, 30, and 35%. Reactions were stopped after 6 h. GOS synthesis by β-glu x and commercially available β-glucosidases (Almond β-glucosidase from Sigma and Novozym 188 from Novo Nordisk A/S, Bagsvaerd, Denmark) was compared using 0.6 U/mL of enzyme, 30% cellobiose, and pH 5.0 for 6 h. Incubation temperatures were 50°C for β-glu x and Novozym 188, and 40°C for Sigma Almond β-glucosidase. Novozym 188 is a soluble commercial preparation of β-1,4-glucosidase (cellobiase) derived from *Aspergillus niger* with an activity of 250 cellobiose units (CBU)/g. According to the manufacturer, the CBU corresponds to the quantity of enzyme that gives 2 µmol of glucose/min from 4.43 m*M* cellobiose during a period of 15 min at 40°C and pH 7.0.

Structural Analysis of GOSs

MS data of the purified oligosaccharide products were obtained by electrospray (MS-ES) measurements. Solvent was ultapure water, and detection was performed in the positive mode (LRTL, INRA de Rennes,

Fig. 1. Analysis of β-glucosidase production in xylose crude extract by native PAGE. The gel was stained with the chromogenic substrate X-glu at 40 µg/mL.

France). ^{13}C nuclear magnetic resonance (NMR) was recorded on a JEOL-JNM LA400 (400 MHz) spectrometer (Laboratoire Commun d'Analyse, Université de La Rochelle), with an internal reference of tetramethylsilane. Samples were studied as solutions in D_2O.

Results

Production and Purification of β-glu x

S. sclerotiorum was grown in liquid culture (shake flasks) at 25°C for 12 d with 1% (w/v) xylose used as carbon source. Analysis of the culture supernatant by native PAGE followed by activity staining with the specific chromogenic substrate X-glu showed that one extracellular enzyme (β-glu x) was produced (Fig. 1). The purification procedure of β-glu x from crude enzyme preparation is detailed in Materials and Methods, and Table 1 summarizes it. In all steps enzymatic tests of β-glucosidase, β-xylosidase, endoglucanase, and xylanase activities were released. The DEAE-Sepharose step allowed a good separation of β-glu x from the others, especially from

Table 1
Purification Steps of β-glu x From *S. sclerotiorum*

Step	Total activity (U)	Total proteins (mg)	SA (U/mg of proteins)	Recovery (%)	Purification (fold)
Crude extract	92	26	3.5	100	1
Ammonium sulfate precipitation	79	19	4.1	85	1.1
Gel filtration (Sephadex G150)	68	9	7.5	73	2.1
DEAE Sepharose CL6B	51	0.42	121.4	55	34.6
HPLC gel filtration	32	0.17	188.2	34	53.7

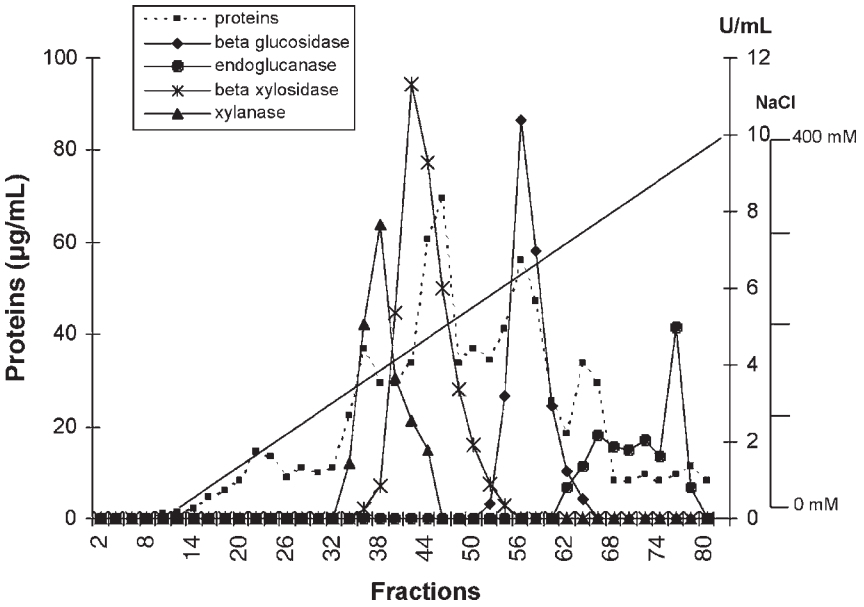

Fig. 2. Elution pattern of DEAE Sepharose column. Elution was performed using an (0–400 m*M*) NaCl linear gradient. Proteins were determined by the method of Bradford *(24)*.

endoglucanase and β-xylosidase activities (Fig. 2). In a final step consisting of an HPLC gel filtration column, β-glucosidase activity was represented by a single protein peak (Fig. 3). Analysis of this fraction by SDS-PAGE showed a single polypeptide of 60 kDa. This β-glucosidase preparation was used for subsequent analysis.

Biochemical Characterization

Native molecular mass was determined in an HPLC gel filtration purification step using standard proteins. β-glu x native mass was esti-

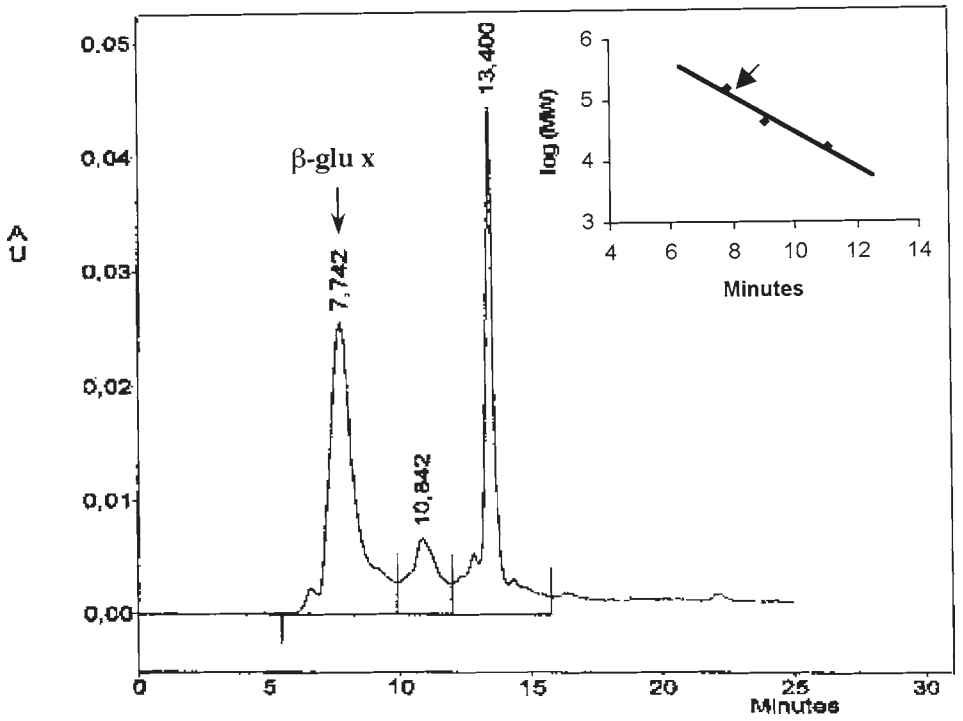

Fig. 3. Elution of β-glucosidase activity from HPLC gel filtration column (TSK 2000SW). Proteins were monitored by determining the absorbance at 280 nm. (Inset) MW curve for standard proteins. AU, absorbance unit; MW, molecular weight.

mated to be 130 kDa. However, SDS-PAGE molecular mass was found to be 60 kDa (Fig. 4), suggesting that the native protein may correspond to a homodimer. The effect of temperature and pH on the activity and stability of β-glu x was studied. The results show that β-glu x had an optimal temperature at 55–60°C, and it retained about 50% of its activity for 30 min at 60°C without substrate. Optimal pH was 5.0, and the enzyme was stable among an interval of pH values, from 4.0 to 7.0. K_m and V_{max} values for pNPGlc were found to be 0.09 m*M* and 193 U/mg, respectively, as determined from a Lineweaver-Burk plot.

The effect of various metallic ions (Co^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}) and other agents (dithiothreitol [DTT], EDTA, glycerol, glucose, δ-gluconolactone) on activity of β-glu x was tested (Table 2). β-glu x was inhibited by glucose, δ-gluconolactone, Zn^{2+}, Cu^{2+}, and Fe^{2+}. In addition, no significant effect on the enzyme activity was observed with DTT, EDTA, glycerol, Co^{2+}, Mg^{2+}, and Mn^{2+}. Finally, the enzyme activity was stimulated by Ca^{2+}.

Synthesis of GOSs

Synthesis was carried out using cellobiose as substrate. Analysis of the reaction mixtures that contained high cellobiose concentration (30% [w/v])

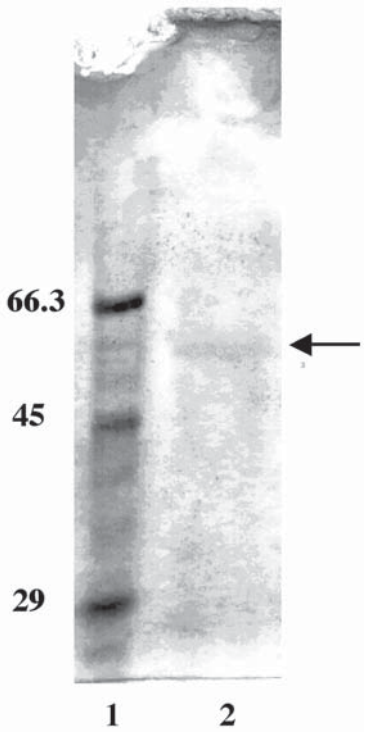

Fig. 4. SDS-PAGE analysis of β-glu x (lane 2) in a 10% polyacrylamide gel stained with silver nitrate. Lane 1, molecular weight markers (BSA: 66.3 kDa; ovalbumin: 45 kDa; carbonic anhydrase: 29 kDa).

Table 2
Effects of Cations and Reagents
on β-glu x Activity From *S. sclerotiorum*

Cation or reagents[a]	Relative activity (%)[b]
None	100
Co^{2+}	78.5
Mg^{2+}	109
Ca^{2+}	135.7
Mn^{2+}	82.6
Zn^{2+}	65.2
Cu^{2+}	36.4
Fe^{2+}	1.2
Glycerol	97.3
Glucose	52.2
δ-Gluconolactone	7.8
DTT	97.6
EDTA	103

[a]All cations or reagents were tested at a concentration of 5 m*M*.

[b]The relative activity was determined by measuring the β-glu x1 activity at 60°C and pH 5.0 after preincubation at 4°C for 30 min in the presence of individual cation or reagent. The activity assayed in the absence of cation or reagent was taken as 100%.

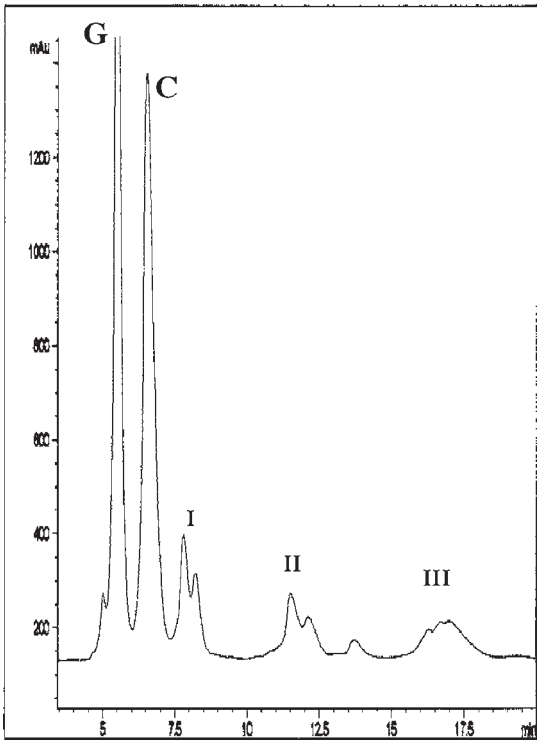

Fig. 5. HPLC analysis of products of GOS synthesis reaction. The reaction was carried out with 30% cellobiose, 0.6 U of enzyme solution at pH 5.0 for 6 h. G, glucose; C, cellobiose; I–III, synthesized GOS.

by HPLC revealed that β-glu x from *S. sclerotiorum* catalyzed hydrolysis and the transglycosylation reactions. As shown in Fig. 5, the HPLC chromatogram displayed five major peaks: G, C, I, II, and III. The cellobiose (substrate) was transformed to generate glucose and three major neosynthesized GOSs (I, II, III). Figure 6 shows the time course of total GOS production with 30% cellobiose at 50°C and pH 5.0 using 0.6 U/mL of enzyme preparation. Cellobiose concentration decreased rapidly during the first 6 h, showing its intense transformation. After incubation for 6 h, the maximum yield of GOSs was attained (27%, 80 g/L). When the reaction time was prolonged, GOSs decreased slowly as they were converted to glucose.

One of the most important factors to increase the yield of transglycosylation products is an increase in substrate concentration, which should make it possible to shift the equilibrium toward the synthesis. The effect of increasing cellobiose concentration on the production of GOSs is shown in Fig. 7. The best production was obtained with 350 g/L (1.02 *M*) cellobiose, reaching 105 g/L.

GOS synthesis level by β-glucosidase from *S. sclerotiorum* (β-glu x) was compared to that by commercially available β-glucosidases: *A. niger*

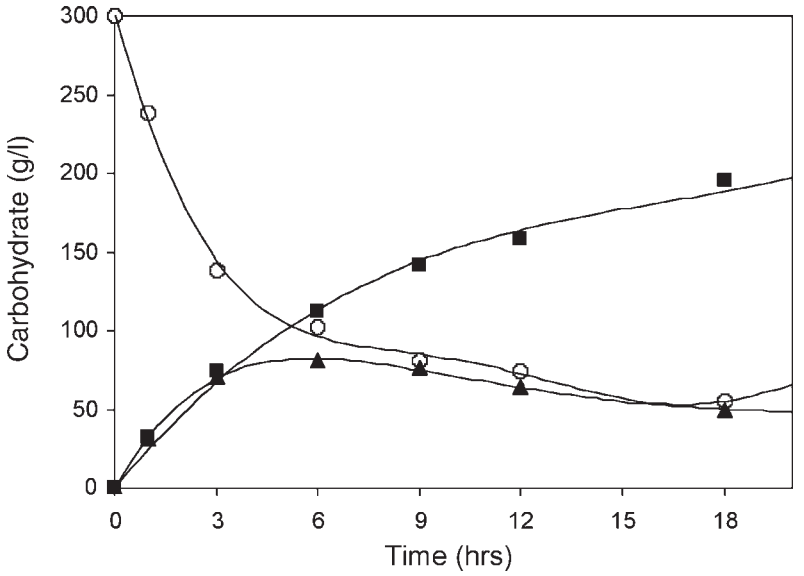

Fig. 6. Time course of synthesis of GOSs by *S. sclerotiorum* β-glu x: (▲) GOSs; (○) cellobiose; (■) glucose.

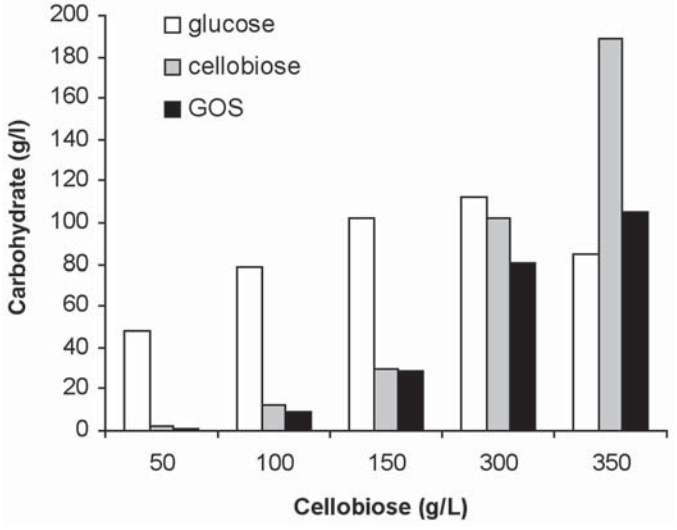

Fig. 7. Effect of increasing cellobiose concentration on GOS production by β-glu x from *S. sclerotiorum*. Reactions were carried out at pH 5.0 and 50°C for 6 h with 0.6 U/mL of enzyme preparation.

(Novo 188) and Almond (Sigma). The reactions were carried out with 300 g/L of cellobiose at pH 5.0 and with 0.6 U of β-glucosidases. β-glu x exhibited the better performance with 80 g/L (27%) of GOS production, while Almond β-glucosidase synthesized only 23 g/L (7.5%) and Novozym 188 only hydrolyzed 89% of the glucose released in the reaction medium.

Table 3
^{13}C NMR Data of Produced GOSs
From Cellobiose With β-glu x From *S. sclerotinia*

Oligosaccharides	Residue[a]	Chemical shift[b] C-1	Chemical shift[b] C-6	Assignment of C-1
I Gentiobiose	α-Glcp I	92.86	69.37	C-1 α
	β-Glcp I	96.70	69.54	C-1 β
	α-Glcp II	103.38	61.45	C-1,6
	β-Glcp II	103.42	61.45	C-1,6
IIa Cellotriose	α-Glcp I	92.95	60.97	C-1 α
	β-Glcp I	96.87	60.98	C-1 β
	Glcp II	103.52	61.66	C-1,4
	Glcp III	103.54	61.67	C-1,4
IIb	α-Glcp I	92.96	60.97	C-1 α
	β-Glcp I	96.89	60.98	C-1 β
	Glcp II	103.52	61.67	C-1,4
	Glcp III	103.54	61.68	C-1,3
III	α-Glcp I	92.24	60.61	C-1 α
	β-Glcp I	96.501	60.74	C-1 β
	α-Glcp II	103.06	69.38	C-1,4
	β-Glcp II	103.07	69.39	C-1,4
	α-Glcp III	103.30	61.30	C-1,6
	β-Glcp III	103.44	61.44	C-1,6

[a]Glcp, glucopyranosyl residue.
[b]In ppm relative to the signal of internal TMSI at 0 ppm in deuterium oxide at 25°C.

To characterize the oligosaccharides produced, the products corresponding to peaks I, II, and III (Fig. 5) were individually collected and analyzed by MS and ^{13}C NMR. MS results showed that the molecular weight of products I, II, and III were 342, 504, and 504 g/mol, respectively. Compound I is consequently a disaccharide while compounds II and III are trisaccharides. The ^{13}C spectrum of oligosaccharides I, II, and III presents several characteristic signals generated by carbons C-1 and C-6 (Table 3). ^{13}C NMR spectroscopy, in agreement with the MS, showed that peak I corresponded to β-Glc-(1→6)-Glc (gentiobiose); peak III corresponded to β-Glc-(1→6)-β-Glc-(1→4)-Glc; and peak II corresponded to two trisaccharides, β-Glc-(1→3)-β-Glc-(1→4)-Glc and β-Glc-(1→4)-β-Glc-(1→4)-Glc (cellotriose).

Discussion

S. sclerotiorum grew well and produced significant levels of β-glucosidase on a wide range of carbon sources. A differential induction was described according to the type of carbon source *(20)*. Two different enzymes (β-glu1 and β-glu2) were produced on filter paper as inducer; they have been purified and characterized *(20)*. One enzyme (β-glu x) was found in the culture filtrate when the fungus grew on xylose as carbon

source. Waksman *(3)* has also described a unique β-glucosidase from *S. sclerotiorum* induced by CMC as the carbon source. This enzyme exhibits different properties compared to β-glu x induced by xylose. These differences may be explained by the different origins of the fungus and by the inducer type. Few works have described the use of xylose as hydrolase inducer. Saha and Bothast *(25)* have studied the effect of various substrates on β-glucosidase production in *Candida peltata*. Interestingly, maximum yield was obtained with xylose. Hrmovà et al. *(26)* showed the induction of cellulose- and xylan-degrading enzymes by xylose, xylobiose, and many artificial xylose-containing disaccharides in *Aspergillus terreus*.

In general, induction of cellulases and other hydrolytic enzymes in fungi is a complicated phenomenon, and despite the large inducers and species tested, a typical and general model is not established.

β-glu x was purified to homogeneity by ammonium sulfate precipitation and three chromatographic steps. Comparison of the native PAGE and SDS-PAGE molecular masses for β-glu x allowed us to conclude that the enzyme is a homodimer. Similar results were found for several fungal β-glucosidases *(20,27–29)*.

By determining the apparent kinetic parameters, we can conclude that β-glu x has a high affinity for pNPGlc (K_m = 0.09 m*M*). In addition, it efficiently hydrolyzes cellobiose (data not shown). Generally, β-glucosidases may be divided into three groups on the basis of substrate specificity: (1) aryl β-glucosidase (which exclusively hydrolyzes 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) *(25,30)*. Thus, β-glu x seems to belong to the third and common group of β-glucosidases.

The effect of temperature and pH on purified β-glu x activity and stability was studied. β-glu x is similar to other fungal β-glucosidases with respect to optimal activity conditions (55–60°C and pH 5.0). The optimal pH and temperatures of β-glucosidases from various microbial sources range between 3.0 and 7.0, and 40 and 105°C, respectively *(31–33)*. The effect of several cations and other reagents was also studied on the pNPGlc hydrolysis activity. The inhibition of β-glucosidases by glucose and δ-gluconolactone is a common phenomenon *(31)*. Inhibition by Zn^{2+}, Cu^{2+}, and Fe^{2+} may be owing to complex formation and/or catalysis of oxidation of specific residues (thiol groups), or nonspecific salt formation *(25,30)*. The chelating agent EDTA did not affect β-glu x activity, indicating that β-glu x is not a metalloprotein. Comparably, DTT did not act as inhibitor, suggesting that disulfide bonds are not essential for enzyme activity. Activation by Ca^{2+} may be explained by the stabilization of the enzyme structure. Hence, it can be useful for β-glu x immobilization.

The transglycosylation of cellobiose by β-glu x from *S. sclerotiorum* was described. The cellobiose was converted into four major GOSs, observed by HPLC analysis: β-Glc-(1→6)-Glc (gentiobiose), β-Glc-(1→6)-β-Glc-(1→4)-Glc, β-Glc-(1→3)-β-Glc-(1→4)-Glc, and β-Glc-(1→4)-β-Glc-

(1→4)-Glc (cellotriose). At high levels of GOSs, 105 g/L from 35% of cellobiose at 50°C, pH 5.0, (6 h) was formed in the reaction medium with the free β-glu x from *S. sclerotiorum*. We have also shown that the amount of GOSs formed with β-glu x from *S. sclerotiorum* was three times higher than the GOSs formed with Almond β-glucosidase tested under the same conditions.

Several articles have described cellobiose transglycosylation with β-glucosidases for tri- and tetraoligosaccharide production. The proposed mechanism is that the enzyme hydrolyzes cellobiose and releases a free glucose. An enzyme-glucose complex may be formed and then the glucose is transferred (several β-glucosidic linkages can be formed) to the substrates presented in the reaction medium to form first trisaccharides and thereafter tetrasaccharies by binding glucose to trisaccharide *(10,14,34,35)*. Thus, the use of cellobiose as substrate augments the triglucoside formation and allows the synthesis of tetraglucosides.

In conclusion, we have described the production by *S. sclerotiorum* of a unique β-glucosidase, β-glu x, induced by an uncommon inducer, xylose, used as carbon source. Its application in GOS synthesis was studied, and the GOS yield was one of the highest yields obtained with purified β-glucosidases.

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