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Purification and Characterization of an Extracellular β-Glucosidase With High Transglucosylation Activity and Stability From *Aspergillus niger* No. 5.1

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Abstract

An extracellular β-glucosidase was extracted from the culture filtrate of *Aspergillus niger* No. 5.1 and purified to homogeneity by using ammonium sulfate precipitation, Chitopearl-DEAE chromatography, and Sephadex G-100 chromatography. The specific activity of the enzyme was enriched 6.33-fold, with a recovery of 11.67%. The enzyme was a monomer and the molecular mass was 67.5 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis and 66.5 kDa by gel-filtration chromatography. The enzyme had optimum activity at pH 6.0 and 60°C and was stable over the pH range of 3.0–9.0. It showed specificity of hydrolysis for *p*-nitrophenyl-β-D-glucoside and cellobiose. The K_m and V_{max} values of the enzyme for cellobiose and salicin were 5.34 m*M*, 2.57 μmol/(mL·s), and 3.09 m*M*, 1.34 μmol/(mL·s), respectively. Both amino acid composition and N-terminal amino acid sequence of the enzyme were determined, which provides useful information for cloning of this enzyme.

Index Entries: β-Glucosidase; *Aspergillus niger*; purification; characterization; kinetic parameters; amino acid.

Introduction

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of β-1,4-glucosidic bonds of a variety of glucosides by

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cleaving β-glucoside linkages from nonreducing terminal ends *(1)*. It widely occurs in bacteria, fungi, plants, and animals. It is an important component of the cellulase system and acts synergistically with endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91) for complete degradation of cellulose. Cellulase systems containing a low level of β-glucosidase have poor saccharifying power because of the inhibition of endoglucanase and cellobiohydrolase by cellobiose *(2)*. Therefore, β-glucosidase plays a critical role in the process of cellulose degradation. Furthermore, the physiological roles postulated for β-glucosidases are extremely diverse *(3)*. Transglucosylation by β-glucosidase is another interesting property, which greatly extends the biotechnological application of this enzyme in the production of some biologically active oligosaccharides and nonionic surfactants, alkyl glucosides *(4–7)*.

To find a suitable β-glucosidase for the production of functional cellooligosaccharides and alkyl glucosides, glycosidase activities in fungal culture filtrates were examined. In the course of screening β-glucosidases from fungal culture filtrates, we found that β-glucosidase from *Aspergillus niger* No. 5.1 had high transglucosylation activity and stability. Based on its unique properties, a rapid purification procedure was developed. In this article, we report the purification and characterization of β-glucosidase from the culture filtrate of *A. niger* No. 5.1.

Materials and Methods

Chemicals

Acrylamide, Coomassie Brilliant Blue G250, and bovine serum albumin (BSA) were purchased from Sigma. Protein molecular mass markers and Sephadex G-100 were obtained from Pharmacia. Chitopearl-DEAE was provided by Fuji Spinning (Tokyo, Japan). All other materials used were of the purest grade commercially available.

Organism and Culture Conditions

A. niger No. 5.1 was obtained from the Culture Collection and Research Center of Microbiology, Beijing Normal University, China. The organism was cultivated in a medium containing cellulose powder as the sole carbon source at 30–32°C and prepared according to the method of Cappellini and Peterson *(8)*. An inoculum of spore suspension of the fungus containing 1.0×10^7 spores was introduced into 50 mL of culture solution. After 5 d of growth, the medium was withdrawn and filtered through a sintered glass filter. The filtrate obtained was designated "culture filtrate" and was used as the extracellular enzyme source.

Assay of β-Glucosidase Activity

A substrate solution was prepared by dissolving 1 mg of D(–)-salicin in 100 mL of 0.05 *M* citrate buffer (pH 4.8). β-Glucosidase activity was

assayed by adding 0.1 mL of the diluted enzyme solution to 1 mL of the substrate solution. After incubating at 50°C for 30 min, enzymatic reactions were terminated and analyzed by adding 3 mL of dinitrosalicylic acid reagent. The glucose liberated was determined by the method of Miller *(9)*. One unit of activity was defined as the amount of enzyme that released 1 μmol of glucose/min under the assay conditions.

Determination of Protein

The proteins in column effluents were detected by absorbance at 280 nm. The protein concentration was measured by the Bradford method with crystalline BSA as the standard *(10)*.

Purification of β-*Glucosidase*

Unless otherwise stated, all purification steps were carried out at ambient temperature (20°C). The enzyme preparations at various stages of purification were concentrated by ultrafiltration with a Diaflo UM10 membrane (Amicon, Danvers, MA).

Step 1: Ammonium Sulfate Precipitation

Solid ammonium sulfate was slowly added to the culture filtrates to a final concentration of 80% and stirred for 4 h at 4°C. The mixture was held at 4°C for 2 h, and then the precipitate was collected by centrifugation (5000*g* for 30 min) and redissolved in sterile distilled water. The protein solution was dialyzed against 1 L of distilled water containing 1 m*M* EDTA at 4°C overnight. The dialysate was collected and used for further purification.

Step 2: Anion-Exchange Chromatography

The dialyzed solution was applied to a column (2.5 × 40 cm) of Chitopearl-DEAE previously equilibrated with 0.2 *M* acetate buffer (pH 3.5). The enzyme was eluted stepwise with three acetate buffers (0.2 *M*, pH 3.5; 0.3 *M*, pH 4.5; 0.4 *M*, pH 5.5) at a flow rate of 1 mL/min. Fractions of 20 mL were collected and monitored for protein concentration and enzyme activity. The major bulk of β-glucosidase was eluted with 0.3 *M* acetate buffer (pH 4.5). Fractions (620 mL) containing most of the β-glucosidase activity were pooled and concentrated to about 20 mL.

Step 3: Sephadex G-100 Gel Filtration

The concentrated enzyme solution was applied to a Sephadex G-100 column (1.5 × 20 cm) previously equilibrated with 50 m*M* acetate buffer (pH 5.0). The column was washed with the same buffer (pH 5.0) at a constant flow rate of 0.1 mL/min. Fractions of 4 mL were collected and analyzed for protein concentration and enzyme activity. A protein peak containing β-glucosidase activity was obtained in this stage. The active fractions were then combined, dialyzed against water, concentrated, and stored as purified enzyme at –20°C.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to check protein purity and to determine the molecular weight of the purified enzyme under denaturing conditions using a 7.5% separating gel and 5% stacking gel containing 0.1% SDS *(11)*. Bromophenol blue was used as the tracking dye. Protein was stained with Coomassie Brilliant Blue G250 and destained with methanol/acetic acid/water. The molecular mass of the subunit was estimated with standard markers.

Determination of Molecular Mass

The molecular mass of the native protein was determined by gel-filtration chromatography on a Sephadex G-100 column (1.6 × 96 cm) at a flow rate of 6 mL/h with a 20 m*M* sodium citrate buffer (pH 5.0) containing 0.5 *M* NaCl as eluate. The molecular mass of the purified β-glucosidase was estimated with marker proteins.

Substrate Specificity of β-Glucosidase

To investigate the substrate specificity of the enzyme, several arylglycosides, disaccharides, and some oligosaccharides were tested in 50 m*M* citrate buffer (pH 6.0) at 60°C for 30 min.

Kinetic Study

The kinetic parameters of the purified β-glucosidase were measured by adding both salicin (0.05–20 m*M*) and cellobiose (0.05–20 m*M*) as substrates into the reaction mixture and by assaying the hydrolysis activity as described previously. The K_m and V_{max} values were calculated from double-reciprocal plots.

Amino Acid Composition

Amino acid analysis of β-glucosidase was performed as follows: The enzyme solution was dried using an SC100A Speedvac Plus (Savant). The samples were hydrolyzed with 6 *M* HCl, 4 *M* methanesulfonic acid for tryptophan in evacuated tubes, and the tubes were sealed under vacuum at 110°C for 22 h using a Waters Pico-Tag Workstation. For analysis of cysteine and methionine, the samples were hydrolyzed with performic acid reagent for 30 min before the addition of 6 *M* HCl. The resulting amino acid compositions were analyzed on an Na high-performance column and calculated by an amino acid analyzer (Beckman System 6300) according to the manufacturer's instructions.

N-Terminal Amino Acid Sequencing

Amino acid sequencing was done with protein samples obtained from SDS-PAGE. The protein in the gel was transferred onto poly-(vinylidene difluoride) membranes in a Mighty Small Transphor Unit (Hoefer TE 22;

Table 1
Purification of β-Glucosidase From *A. niger* No. 5.1[a]

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme	240.00	5923.2	24.68	1.00	100.00
$(NH_4)_2SO_4$	60.55	2516.22	41.56	1.68	42.48
Chitopearl-DEAE	6.68	845.42	126.56	5.13	14.27
Sephadex G-100	4.42	690.98	156.33	6.33	11.67

[a]Activities were determined using D(–)-salicin as substrate. Purification factor = specific activity/24.68; yield = (total activity/5923.2) × 100%. Diethylaminoethyl (DEAE).

Pharmacia-Biotech, Uppsala, Sweden). The transferred protein was visualized by Ponceau S staining and excised with a razor blade. N-Terminal amino acid sequencing was performed on an Applied Biosystems model 477A gas-phase sequencer equipped with an automatic on-line phenylthiohydantoin amino acid analyzer.

Results

Purification of β-*Glucosidase*

The results of the purification procedure for β-glucosidase produced are summarized in Table 1. The culture filtrate of *A. niger* No. 5.1 was subjected to ammonium sulfate precipitation. The recovery of β-glucosidase was 42.48% in this step. On Chitopearl-DEAE column purification of the ammonium sulfate–precipitated fraction, the enzyme was purified 5.13-fold with a recovery of 14.27%. The final purification step was conducted by charging the Chitopearl-DEAE eluate onto a Sephadex G-100 column. β-Glucosidase with an activity of 156.33 U/mg of protein was eluted as a single peak and purified 6.33-fold with a recovery of 11.67%.

General Properties

As shown in Fig. 1, the purified enzyme was visible as a single band in SDS-PAGE. Accordingly, the enzyme was considered to be purified to homogeneity. The molecular mass of the enzyme was estimated to be 67.5 kDa by comparison of its relative mobility on SDS-PAGE with those of standard proteins. The molecular mass was also determined by gel filtration, and the enzyme was eluted at the position corresponding to a molecular mass of approx 66.5 kDa (Fig. 2). These results suggest that the nondenatured enzyme occurs as a monomer.

Figure 3 shows that the optimum pH of the β-glucosidase was 6.0. The enzyme was stable over the pH range of 3.0–9.0 after preincubation at 50°C for 60 min. The activity of the enzyme still remained 75 and 60% of the original activity at pH 2.0 and 10.0, respectively. Figure 4 shows that the optimum temperature of the catalytic reaction was at 60°C, and that

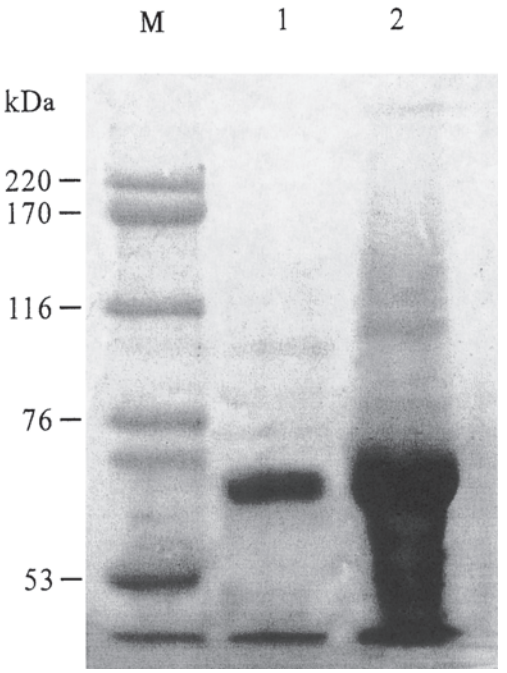

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of purified β-glucosidase. M, marker proteins; lane 1, purified enzyme from Sephadex G-100 column; lane 2, crude enzyme from ammonium sulfate precipitation.

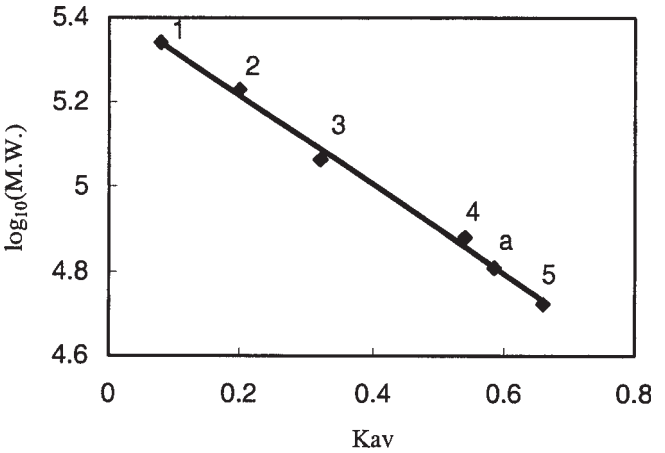

Fig. 2. Determination of molecular mass of β-glucosidase by gel filtration. Standard proteins: 1, rabbit muscle myosin (220 kDa); 2, bovine plasma α_2-macroglobulin (170 kDa); 3, *Escherichia coli* β-galactosidase (116 kDa); 4, human transferrin (76 kDa); 5, glutamic dehydrogenase (53 kDa). a, purified β-glucosidase.

β-glucosidase activity was stable between 0 and 80°C after preincubation at pH 6.0 for 60 min. However, about 60% of the original activity was lost after preincubation at 90°C for 60 min.

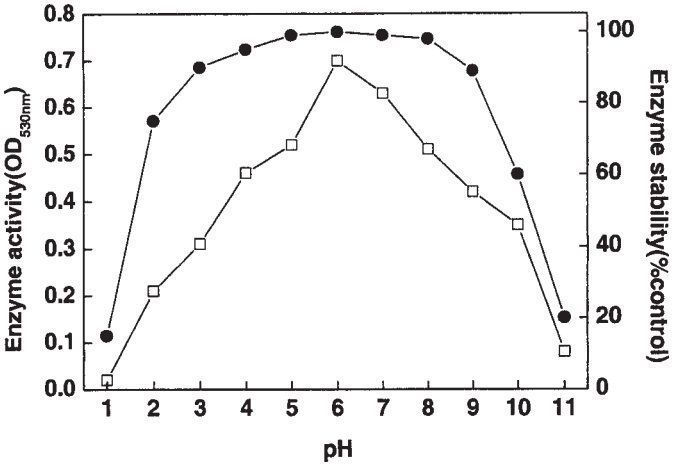

Fig. 3. Effect of pH on activity and stability of β-glucosidase: (□) enzyme activity at 50°C; (●) pH stability: preincubation at 50°C for 60 min.

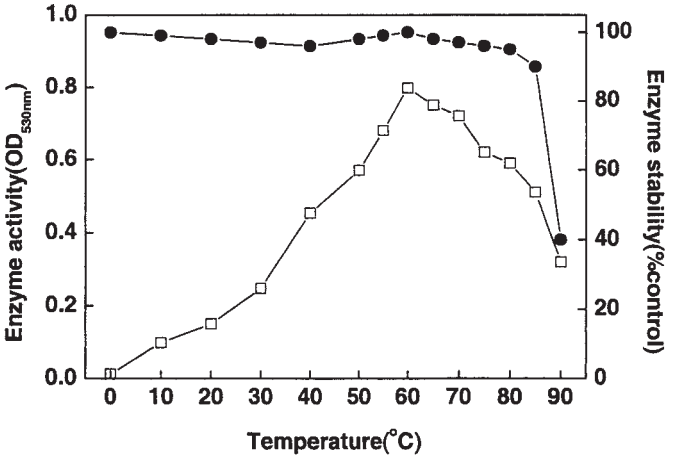

Fig. 4. Effect of temperature on activity and stability of β-glucosidase: (□) enzyme activity at pH 6.0; (●) thermal stability: preincubation at pH 6.0 for 60 min.

The effect of various metal ions on β-glucosidase activity was also investigated. Among the metal ions tested, Fe^{2+} and Ag^{+} were strong inhibitors. Interestingly, enzyme activity was enhanced in the presence of K^{+}, Na^{+}, Mg^{2+}, and Zn^{2+}. Other metal ions had little or no inhibitory effect on enzyme activity.

Substrate Specificity and Kinetic Parameters

β-Glucosidase-catalyzed hydrolysis of various substrates was studied at optimum pH and temperature, and the corresponding K_m and V_{max} values were determined. Table 2 shows the substrate specificity of the enzyme toward several arylglycosides, disaccharides, and some oligosaccharides.

Table 2
Substrate Specificity of *A. niger* No. 5.1 β-Glucosidase

Substrate	Specific activity (U/mg)	Relative activity (%)
*p*NPG	456.25[a]	100
*p*NPX	91.25[a]	20
D(–)-Salicin	156.33[b]	87
Cellobiose	179.69[b]	100
Cellotriose	158.17[b]	88
Cellotetraose	134.77[b]	75
Cellopentaose	109.61[b]	61
Crystalline cellulose	0.00[b]	0
Esculin	5.39[b]	3

[a]Specific activity is reported as micromoles of *p*-nitrophenol released per minute per milligram of protein.

[b]Specific activity is reported as micromoles of glucose equivalent released per minute per milligram of protein.

*p*NPG, *p*-nitrophenyl-β-D-glucoside; *p*NPX, *p*-nitrophenyl-β-D-xylose.

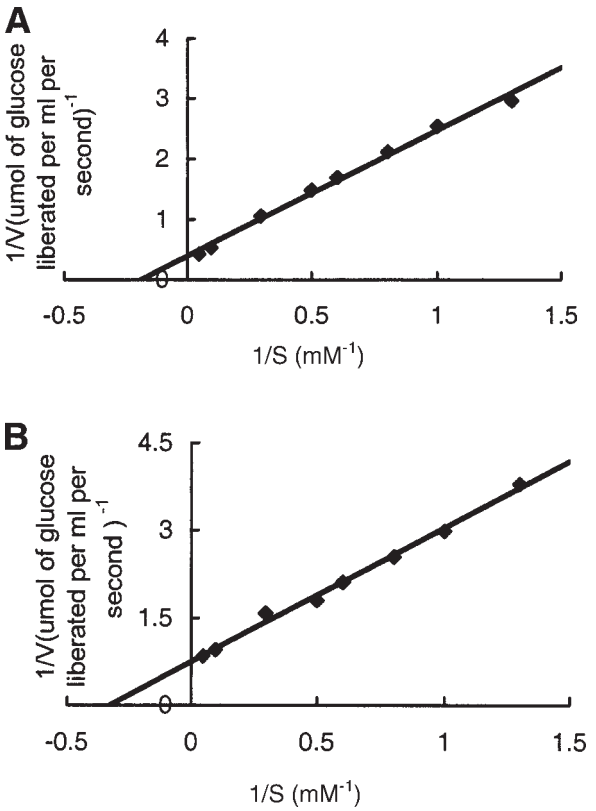

Fig. 5. Lineweaver-Burk plots of β-glucosidase from *A. niger* No. 5.1: **(A)** cellobiose; **(B)** D(–)-salicin. Cellobiose or salicin: 1.0 mL of 0.05–20 m*M*; citrate buffer: 2.0 mL of 0.05 *M*, pH 6.0; enzyme: 1 mL (1 mg/mL); incubation: 60°C, 30 min.

Table 3
Amino Acid Composition
of β-Glucosidase From *A. niger* No. 5.1

Amino acid	Composition molar ratio (%)
Glycine	19.67
Glutamic acid	14.32
Alanine	12.83
Aspartic acid	9.94
Isoleucine	8.78
Valine	6.08
Serine	5.02
Proline	4.43
Tyrosine	3.95
Lysine	3.83
Tryptophan	3.05
Threonine	2.25
Histidine	2.01
Phenylalanine	1.50
Leucine	1.27
Methionine	0.63
Cysteine	0.36
Arginine	0.08

The enzyme hydrolyzed *p*-nitrophenyl-β-D-glucoside (*p*NPG) and cellobiose more efficiently than *p*-nitrophenyl-β-D-xylose (*p*NPX), cellotriose, cellotetraose, cellopentaose, and esculin but was unable to hydrolyze crystalline cellulose. The hydrolysis activity toward the cellooligosaccharides decreased with an increase in subunit number. The kinetic parameters for cellobiose and D(–)-salicin were calculated from Lineweaver-Burk plots (Fig. 5). The K_m and V_{max} values of the enzyme for cellobiose and D(–)-salicin were 5.34 m*M*, 2.57 μmol/(mL·s) and 3.09 m*M*, 1.34 μmol/(mL·s), respectively.

Amino Acid Composition and N-Terminal Sequence of β-*Glucosidase*

The amino acid composition of the purified β-glucosidase shows that the most abundant amino acid resides were glycine (19.67%), glutamic acid (14.32%), and alanine (12.83%) (Table 3). The N-terminal amino acid sequence of β-glucosidase was determined to be SSPGAYSEAGYGPSDPHGAV, which provides useful information for further gene cloning of this enzyme.

Discussion

We identified a novel β-glucosidase with wide pH and thermal stabilities. The enzyme was purified to homogeneity by a simple and rapid method. First, 80% saturation of ammonium sulfate was added to the cul-

ture filtrate to obtain the crude protein precipitate. Second, β-glucosidase was absorbed onto a Chitopearl-DEAE (with a highly macroporous crosslinked chitosan support) column under the used conditions and was eluted easily from the column with a gradient elution of different concentration and different pH. Third, multiple impurities with low molecular weight were efficiently excluded by gel filtration on a Sephadex G-100 column. In contrast to other methods reported by researchers *(12–14)*, the method of isolation and purification of β-glucosidase proposed in our study was simpler.

The purified β-glucosidase preparation was homogeneous on SDS-PAGE. The molecular mass of the enzyme was 67.5 kDa by SDS-PAGE and 66.5 kDa by gel filtration, suggesting that the nondenatured enzyme was most probably a structural monomer. The molecular mass is lower than that of the reported β-glucosidases purified from *A. niger (15–17)*.

The optimum pH (6.0) of the purified enzyme is similar to that of *Flavoba cterium (18)*, and higher than that of *Aspergillus ornatus* (4.6) *(19)* and *A. niger* CCRC 31494 (5.0) *(20)*. The enzyme had a wide pH range of 3.0–9.0 for its activity. The pH stability (3.0–9.0) of the enzyme is beyond the range of most fungal β-glucosidases. Only the pH stability of the β-glucosidases from *Aspergillus fumigatus* (2.0–8.0) *(21)* is near that of our data. The optimum temperature (60°C) of the purified enzyme is similar to that of *Aspergillus aculeatus (22)*, and lower than those of the other fungal enzymes. However, the thermal stability (up to 80°C for 60 min at pH 6.0) of the purified enzyme is beyond the range of values reported for this enzyme from other *Aspergillus* species.

The inactivation of β-glucosidase by the addition of salts of heavy metals, especially of mercury, silver, and copper, is well known *(23)*. This inactivation might be owing to nonspecific salt formation with the enzyme, since the activity of fungal β-glucosidase was also affected by these chemicals. The inhibitory effect of various metals on enzyme activity is controversial. Mandels and Reese *(23)* presented a table that shows wide discrepancy as to which metals are inhibitory. Well-known inhibitors, such as silver and copper, are listed among the inactive group. These differences may be owing to variations in experimental conditions, such as pH, ionic concentration of buffer, concentration of enzyme, and presence of impurities in the solution. The purified enzyme was also inhibited by heavy metal ions such as Ag^{+} and Fe^{2+}. However, Cu^{2+} had little effect on it.

Among the substrates tested, *p*NPG was the best substrate for the enzyme, followed by cellobiose, cellotriose, salicin, cellotetraose, cellopentaose, and *p*NPX. The enzyme is unable to hydrolyze α-linkages, but, as is the case for some other fungal enzymes, it has partial β-xylosidase activity *(19)*. A broad range of K_m values for salicin and cellobiose has been reported from different fungal sources, including *Aspergillus* species *(24–26)*. This may be attributed to different conditions employed for its determination. The kinetic properties of the purified β-glucosidase revealed that the enzyme had a higher affinity toward salicin than cellobiose, but the

maximum reaction rate of the enzyme toward cellobiose was higher than that of the salicin. Therefore, β-glucosidase is considered a cellobiase with aryl-β-glucosidase activity.

The N-terminal amino acid sequence of the purified enzyme was compared with the sequences of other β-glucosidases. Significant similarity was found between the *A. niger* β-glucosidase and β-glucosidases from *Aspergillus wentii (27)*, *Saccharomyces fibuligera (28)*, *Schizophyllum commune (29)*, and *Prunus serotina (30)*. However, no significant identity was found between the *A. niger* β-glucosidase and those enzymes. This reveals that the purified enzyme is different from other reported β-glucosidases.

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