Purification and Characterization of an Extracellular β-Glucosidase II with High Hydrolysis and Transglucosylation Activities from *Aspergillus niger*

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An extracellular β-glucosidase II (β-Glu II) has been purified to homogeneity by column chromatography from *Aspergillus niger* CCRC 31494. Its molecular mass was estimated to be 360 kDa by gel filtration and 120 kDa by SDS−PAGE. The enzyme has a p*I* of 4.0 and has optimum activity at pH 4.5 and 60 °C. The β-Glu II was completely inhibited by 5.0 mM Fe^{2+}. Methanol (20%, v/v) activated the enzyme activity at 1.8-fold. V_{max} values of 10.2 and 464 units/mg were found for *p*-nitrophenyl β-D-glucoside (K_m = 2.2 mM) and for cellobiose (K_m = 15.4 mM). The enzyme was strongly inhibited by substrates, *p*-nitrophenyl β-D-glucopyranoside in excess of 7.5 mM and cellobiose in excess of 50 mM, and was competitively inhibited by glucose with a K_i of 5.7 mM. Transglucosylation products of cellotriose, methyl β-glucoside and ethyl β-glucoside, were obtained under neutral conditions and in the presence of methanol and ethanol, respectively.

Keywords: *β-Glucosidase; Aspergillus niger; purification; kinetics; transglucosylation*

INTRODUCTION

β-Glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of glycosidic linkages in aryl and alkyl β-D-glucosides as well as cellobiose and occurs widely in plants, fungi, animals, and bacteria. The enzyme is well-known as a component of cellulase complex and has a synergistic action on the degradation of cellulose with endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91). The β-glucosidase not only catalyzes the final step of cellulose degradation but also stimulates the extent of cellulose hydrolysis by relieving the inhibition of exoglucanase and endoglucanase from cellobiose (Shewale, 1982). The physiological roles postulated for β-glucosidases are extremely diverse (Esen, 1993). Besides, transglucosylation by the β-glucosidase is an 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 (Christakopoulos et al., 1994; Fujimoto et al., 1988; Sasaki et al., 1989; Shinoyama et al., 1991)

Among biological sources, the fungus *Aspergillus niger* is regarded as a good β-glucosidase producer. Both intracellular and extracellular enzymes have been purified and characterized. The purified enzymes show remarkable similarities in structural and enzymatic properties. However, the physical, chemical, and functional properties of β-glucosidases vary from species to species (McCleary and Harrington, 1988; Sanyal et al., 1988; Unno et al., 1988; Watanabe et al., 1992; Woodward and Wiseman, 1982; Workman and Day, 1982; Yeoh et al., 1986, 1988). To find a suitable β-glucosidase for the production of functional cello-oligosaccharides and alkyl glucosides, glycosidase activities in fungi culture filtrates were examined. On screening β-glucosidases from the culture filtrate of *A. niger* CCRC 31494, we found three different enzyme activity bands on native polyacrylamide gel electrophoresis (PAGE) with an activity stain. The three enzymes were tentatively named β-glucosidase I (β-Glu I), β-glucosidase II (β-Glu II), and β-glucosidase III (β-Glu III), according to the mobility of each enzyme on native-PAGE. The rapid mobility one, β-Glu I, has a low p*I* near pH 3.0 with a highly glucose-tolerant property, which has been purified and characterized (Yan and Lin, 1997). The middle one, β-Glu II, with a major *p*-nitrophenyl β-glucopyranoside (pNPG) hydrolysis activity, was purified by column chromatography. The purified β-Glu II has high transglucosylation activity as well as hydrolytic activity that can be applied for cello-oligosaccharides and alkyl glucoside production. In this paper, we report the purification and characterization of the β-Glu II from the culture filtrates of *A. niger* CCRC31494.

MATERIALS AND METHODS

Chemicals. pNPG, other *p*-nitrophenyl derivatives, cellobiose, cellotriose, cellotetrose, cellopentose, other disaccharides, and natural substrates were purchased from Sigma Chemical Co. (St. Louis, MO). Sephacryl S-300, PhastGels, buffer strips, and molecular mass markers for gel filtration and for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and low p*I* marker for IEF−PAGE were obtained from Pharmacia-LKB Biotechnology (Uppsala, Sweden). Ampholyte for isoelectric focusing (IEF) was obtained from Bio-Rad Laboratories (Richmond, CA). Unless specified otherwise, all chemicals were of analytical grade.

Organism and Cultivation Conditions. *A. niger* CCRC31494 was purchased from the Culture Collection and Research Center of the Food Industry and Research Development Institute, Hsinchu, Taiwan. The composition of the culture medium, cultivation conditions and preparation of the culture filtrate were followed as described previously (Yan and Chiou, 1996).

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Protein Determination. Protein content was measured according to the method of Bradford (1976) using bovine serum albumin as a standard. The absorbance at 280 nm was used for monitoring the protein in column effluents.

N-Terminal Amino Acid Sequencing Analysis. 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, 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 phenylthiohydrantoin (PTH) amino acid analyzer.

Assay of β-Glu II Activity. The enzyme activity toward pNPG was measured by the amount of *p*-nitrophenol liberated from pNPG using a calibration curve at 410 nm (McCleary and Harrington, 1988). The reaction mixture containing 0.2 mL of suitable enzyme solution and 0.2 mL of substrate (20 mM) in a 100 mM sodium citrate buffer (pH 4.0) was incubated at 40 °C for 10 min. The reaction was then terminated and color developed by the addition of 3 mL of aqueous sodium carbonate (2.0%, w/v). The absorbance at 410 nm was measured and the amount of the released *p*-nitrophenol determined by reference to a *p*-nitrophenol standard curve (prepared in 2%, w/v, aqueous sodium carbonate). The activity against the other *p*-nitrophenyl glycosides was assayed according to the same procedure. One unit of β-glucosidase activity was defined as the amount of enzyme required to catalyze the formation of 1.0 μmol of *p*-nitrophenol/min.

The enzyme activity toward nonchromogenic substrates (cellobiose and oligosaccharides) was determined on the basis of D-glucose liberated. A reaction mixture containing 0.2 mL of the enzyme solution and 0.2 mL of 10 mM substrate in 100 mM sodium citrate buffer (pH 4.0) was incubated at 40 °C for 2 h, and the released D-glucose was measured using the glucose oxidase/peroxidase method (Huggest and Nixon, 1957). One unit of enzyme activity was defined the amount of the enzyme required to catalyze the formation of 1.0 μmol of glucose equivalent/min.

Purification of β-Glu II. All operations were carried out at 4 °C.

Step 1. Ethanol Fractionation. The concentrated culture filtrate (20 mL) containing 41.6 mg of protein was fractionated by adding ice-cold ethanol to a final concentration of 70% (v/v), and then the mixture was centrifuged at 8000*g* for 30 min. The precipitated proteins were recovered and dissolved in 10 mL of 20 mM sodium acetate buffer (pH 4.5) for further purification.

Step 2. First CM-Sepharose Column Chromatography. The enzyme fraction just obtained was applied to a CM-Sepharose fast flow column (2.6 × 30 cm) that had been pre-equilibrated with 20 mM sodium acetate buffer (pH 4.5). The column was washed with 400 mL of the same buffer with a flow rate of 100 mL/h, and 5.0-mL fractions were collected. The enzyme activity was obtained in unbound fractions. The fractions (no. 50−62, 57 mL) containing the enzyme activity were pooled and dialyzed against 20 mM sodium acetate buffer (pH 4.0) at 4 °C for 24 h.

Step 3. Second CM-Sepharose Column Chromatography. The dialyzed enzyme fraction was applied to another pre-equilibrated CM-Sepharose fast flow column (2.6 × 30 cm) with 20 mM sodium acetate buffer (pH 4.0). The column was washed with 400 mL of the same buffer, and the enzyme activity was eluted with a linear gradient of 0−0.5 M NaCl in the sodium acetate buffer (pH 4.0, 500 mL). The flow rate was 100 mL/h, and each fraction of 5.0 mL was collected. The fractions (no. 135−165, 150 mL, 0.2−0.4 M NaCl) containing the enzyme activity were pooled, concentrated to 30 mL, and then dialyzed against 20 mM sodium acetate buffer (pH 4.5) at 4 °C for 24 h.

Step 4. Q-Sepharose Column Chromatography. The enzyme fraction obtained by the second CM-Sepharose fast flow column was applied to a Q-Sepharose fast flow column (2.6 × 30 cm) that had been pre-equilibrated with 20 mM sodium acetate buffer (pH 4.5). The column was washed with the same buffer (pH 4.5), and the enzyme activity was eluted with a linear gradient of 0−0.5 M NaCl in the same buffer (pH 4.5, 500 mL). The flow rate was 100 mL/h, and fractions of 5.0 mL were collected. The fractions (no. 209−214, 20 mL, 0.3-0.4 M NaCl) containing the enzyme activity were pooled and dialyzed against 20 mM sodium acetate buffer (pH 5.0) at 4 °C for 24 h.

Step 5. Sephacryl S-300 HR Gel Filtration. The dialyzed enzyme active fraction was concentrated to a final volume of ∼20 mL before being applied to a preparative Sephacryl S-300 HR gel filtration column (5.0 × 100 cm) that had been pre-equilibrated with 2000 mL of 20 mM sodium acetate buffer (at pH 5.0) containing 0.1 M NaCl. Elution was performed with 1500 mL of the same buffer at the flow rate of 150 mL/h, and fractions of 15 mL were collected to assay for the enzyme activity. The enzyme active fractions (no. 51−59, 57 mL) were dialyzed against 20 mM sodium acetate buffer (pH 4.0) for 24 h and stored as the purified enzyme.

Polyacrylamide Gel Electrophoresis. The purity of the enzyme was determined by nondenaturing polyacrylamide gel electrophoresis (10% T and 2.65% C), (Laemmli, 1970) using the PhastSystem (Pharmacia). Protein was stained with Coomassie Brilliant Blue. For β-glucosidase activity stain, a non-acid fixed gel was soaked in a 100 mM sodium citrate buffer (pH 5.0) containing esculin ferric chloride for 5.0 min. The gel was incubated at 50 °C for several minutes until a black band appeared (Kwon et al., 1994). The subunit composition of the enzyme was determined by SDS−PAGE on a 10−15% gradient PhastGel (Weber and Osborn, 1969). Molecular mass of the subunit was estimated with standard markers.

p*I* Determination. Isoelectric focusing was performed according to the method of Robertson et al. (1987) using a polyacrylamide gel (5% T and 3.3% C) with Pharmalyte (pH 2.5−5.0) as a carrier. The standard low p*I* marker kit (pH 2.5−6.5) was used.

Estimation of Molecular Mass. The molecular mass of native protein was determined by gel permeation chromatography on a Sephacryl S-300 column (2.6 × 95 cm) at a flow rate of 20 mL/h with a 20 mM sodium citrate buffer containing 0.1 M NaCl (pH 5.0) as eluate (Andrews, 1965). The molecular mass of the purified β-glucosidase was estimated with standard markers.

Kinetic Study. The kinetic parameters of the purified β-glucosidase were carried out by adding either pNPG (0.03−20 mM) or cellobiose (0.03−20 mM) as substrate into the reaction mixture and assaying hydrolysis activity as described previously. The K_m and V_{max} values were calculated from double-reciprocal plots. The k_{cat} value was calculated as V_{max} divided by the molar concentration of the enzyme used. The inhibition of glucose or other hexoses on enzyme activity was determined by adding various concentrations of glucose or hexoses into reaction mixtures with pNPG as substrate. The inhibition constant (K_i) was obtained at the intersection of the line of a Dixon plot.

Transglucosylation Study. Under neutral conditions, 0.2 mL of substrate solution (20 mg/mL of cellobiose in 100 mM sodium acetate buffer, pH 5.0) was added to 0.2 mL of the enzyme solution (0.25 unit/mL) and incubated at 40 °C for 10, 20, and 30 min, respectively. The reaction mixture was heated in boiling water for 1 min and then filtered. Each reaction mixture sample (5 μL for TLC and 20 μL for HPLC) was taken and analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). TLC was performed as follows. Samples were spotted on silica gel (60 F254) plates and developed by the ascending method with 2-propanol/acetic acid/water (7:1:2, v/v). Sugars on developed plates were determined by heating at 120 °C after spraying with 10% H_2SO_4. Transglucosylation products were identified by HPLC over a LiChrosphere 100 NH_2 column (E. Merck, Darmstadt, Germany) eluted with acetonitrile/water (80:20, v/v) as mobile phase at a flow rate of 1.0 mL/min with a Jusco 880-PU programmable pump. Elution was monitored by a differential refractometer (RI-930, Jusco, Japan).

Table 1. Purification of *A. niger* β-Glu II

	vol (mL)	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	purification (-fold)
crude enzyme	20	41.6	675	16.2	100	1.0
ethanol fractionation	30	36	614	17.0	91	1.0
1st CM-Sepharose	57	31	536	17.3	79	1.1
2nd CM-Sepharose	30	6.8	245	36	36	2.0
Q-Sepharose	20	1.7	188	112	28	7.0
Sephacryl S-300	123	0.41	150	366	22	23

In the presence of alcohol, 0.08 mL of 30% of methanol or ethanol and 0.2 mL of the cellobiose substrate solution (30 mg/mL) were added to 0.12 mL (0.25 unit/mL) of the purified enzyme solution and incubated at 40 °C for 30 min. After the reaction mixture was heated in boiling water for 1 min to inactivate the enzyme, the reaction products were analyzed by TLC and HPLC with the procedures described above.

RESULTS

Purification. A summary of the purification procedure results for the extracellular β-Glu II produced by *A. niger* CCRC 31494 is presented in Table 1. In the second CM-Sepharose column chromatography, the enzyme was adsorbed onto the column and eluted at 0.3−0.4 M NaCl. Through this step, the enzyme was purified by 2.0-fold with a recovery of 36% of the initial activity (Figure 1A and Table 1). The Q-Sepharose column chromatography increased the purity by 7-fold and gave a recovery of 28% (Figure 1B and Table 1). At the final step, the β-Glu II was eluted as a single peak coinciding with its activity peak, which had a specific activity of 366 units/mg (Figure 1C and Table 1). Finally, the enzyme was purified 23-fold with a recovery of 22% (Table 1). The purified enzyme showed a single protein band on native-PAGE (Figure 2A, lane a), which coincided with activity in activity stain (Figure 2A, lane b). The enzyme also showed a single protein band on SDS−PAGE (Figure 2B) and on IEF−PAGE (Figure 2C). Accordingly, the enzyme is considered to be purified to homogeneity.

General Properties. The molecular mass of the β-Glu II was determined to be 360 kDa by Sephacryl S-300 gel filtration (Figure 3) and 120 kDa by SDS−PAGE (Figure 2B). Therefore, it is considered to be a trimer. The p*I* of the enzyme was determined to be 4.0 by IEF−PAGE (Figure 2C). The purified β-Glu II was subjected to N-terminal sequence analysis. The 19 N-terminal amino acid sequence was SELAYSPPYYPS-PEANGQG.

The optimum pH of the purified β-Glu II was between 4.0 and 5.0. The enzyme was stable in the pH range of 4.0−6.5. Its activity quickly decreased at pH values >7.0 and <4.0. The optimum temperature of the β-Glu II was at 60 °C. The enzyme was stable below 60 °C and lost half of its activity when incubated at 70 °C for 10 min.

The purified β-Glu II was completely inhibited by 5.0 mM Fe^{2+}, and ∼50% of its activity was inhibited by 5.0 mM of Fe^{3+} and Ag^{+}, respectively. However, Ca^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} had little or no inhibitory effect. Some of the specific chemical reagents did not affect the enzyme activity. They are as follows: EDTA, *o*-phenanthroline, dithiothreitol, cysteine, 2-mercaptoethanol, iodoacetic acid, and *p*-chrolomercuribenzoate.

The effects of some organic solvents on β-Glu II activity are shown in Table 2. The enzyme was activated by methanol (20%, v/v) to 1.8-fold, by ethanol (30%, v/v) to 1.5-fold, by 1-propanol (20%, v/v) to 1.4-fold, by 2-propanol (20%, v/v) to 1.2-fold, by 1-butanol (20%, v/v) to 1.2-fold, by 2-butanol (20%, v/v) to 1.3-fold, and by cyclohexanol (40%, v/v) to 1.2-fold. However, the enzyme activity was inhibited at the high concentrations of alcohols. Besides, the enzyme activity was also inhibited by acetone, acetonitrile, dimethyl sulfoxide, and *N*,*N*-dimethylformamide.

Substrate Specificity and Kinetic Parameters. The enzyme hydrolyzed pNPG, cellobiose, esculin, salicin, arbutin, and methyl β-D-glucoside (Table 3) but could not hydrolyze maltose, lactose, cellotriose, the glucosides of α-linkages, p-NP-β-Xyl, p-NP-β-Gal, p-NP-β-Fru, and p-NP-β-Ara. Even the p-NP-β-cellobiose was only slightly hydrolyzed at the standard assay conditions. According to parts A and B of Figure 4, the V_{max} values of the enzyme for pNPG (K_m = 2.2 mM) and cellobiose (K_m = 15.4 mM) were 10.5 and 464 units/mg, respectively. The k_{cat} values of the pNPG and cellobiose were calculated to be 5.5×10^4 and 1.67×105 min^{-1}, respectively. Substrate inhibition of the enzyme was also observed in Figure 4. When the concentration of pNPG is in excess of 7.5 mM, enzyme activity was decreased (Figure 4A). In the case of cellobiose, when its concentration is >50 mM, the enzyme activity was also decreased (Figure 4B). Glucose showed a competitive inhibition on the enzyme using pNPG (Figure 5) as substrate. The K_i value was calculated to be 5.7 mM, while fructose, galactose, mannose, xylose, and glucosamine did not inhibit the enzyme activity even at 30 mM.

Transglucosylation Activity. Incubation of the enzyme with cellobiose under neutral conditions gave transglucosylation products (analyzed by TLC) showing that the enzyme not only released glucose but also catalyzed the formation of cellotriose, which was also identified by HPLC. Incubation of the enzyme with cellobiose as substrate in the presence of methanol and ethanol gave products of methylglucose and ethylglucose other than triose (Figure 6), which were identified by HPLC. In a preliminary study, the yields of methyl glucoside and ethyl glucoside were calculated to be 82% and 55% (mole/mole, based on cellobiose consumed), respectively.

DISCUSSION

An extracellular β-Glu II from *A. niger* CCRC 31494 was purified to homogeneity with a recovery of 22% of the original activity. The first step (70% ethanol fractionation) and the second step (first CM-Sepharose column chromatography) are used for separating the β-Glu II from an acidic β-glucosidase. Hence, there is no obvious increase in purification fold. The specific activity of the purified enzyme was 366 units/mg of protein under the standard assay conditions described under Materials and Methods and 1071 units/mg of protein under optimum conditions (the same conditions as the standard assay except that reaction temperature

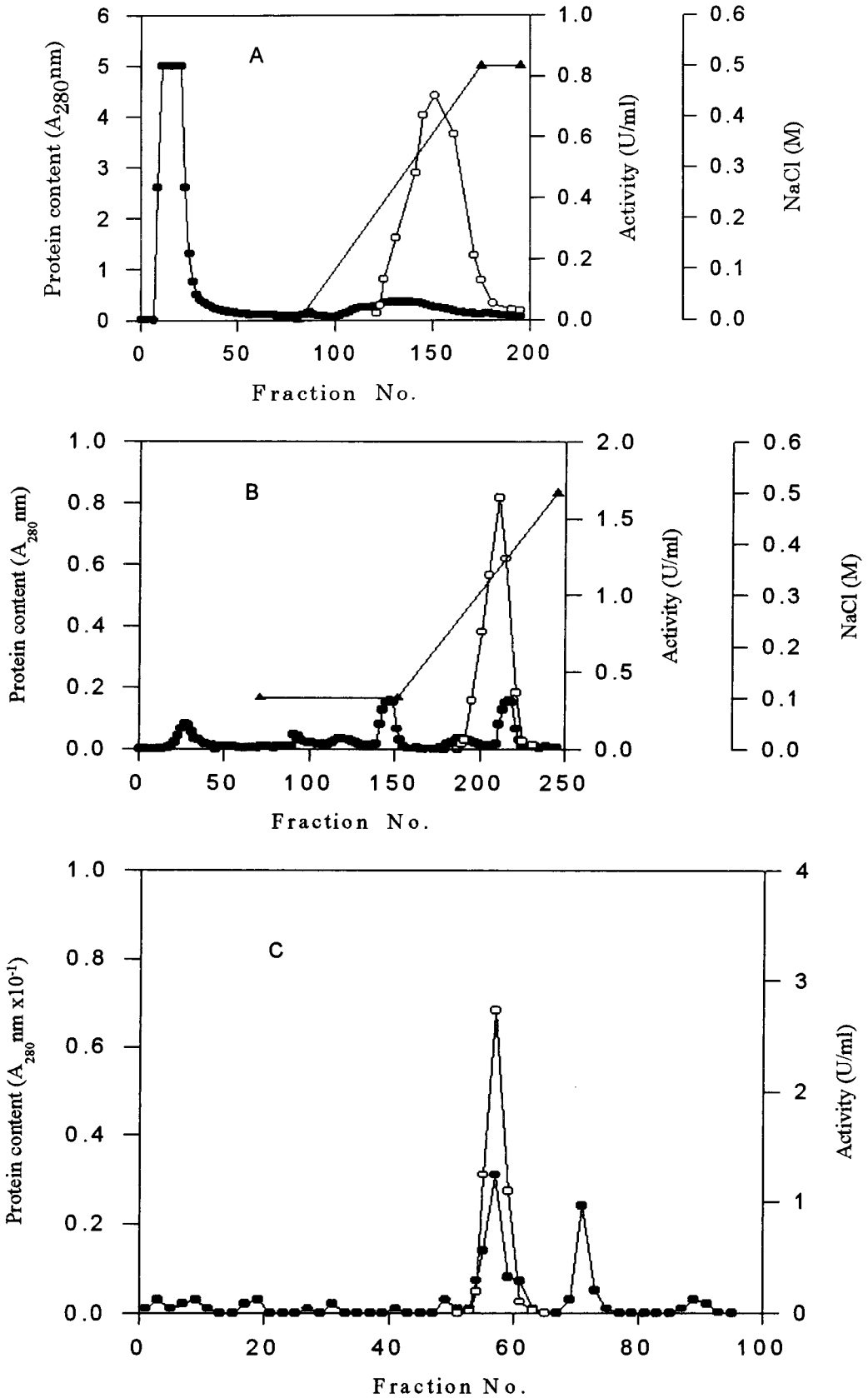

Figure 1. Purification of β-Glu II from *A. niger*: (A) second CM-Sepharose chromatography; (B) Q-Sepharose chromatography; (C) Sepharcryl S-200 HR chromatography. ●, ○, and △ represent protein (A_{280}), β-Glu II activity, and the NaCl gradient, respectively.

and pH were changed to 60 °C and 5.0, respectively). The specific activities of purified β-glucosidases from various microorganisms examined by other researchers varied from 5 to 979 units/mg of protein. Therefore, the specific activity of the purified β-Glu II is similar to the highest one among those recorded enzymes. The molecular mass of the β-Glu II (360 kDa) is larger than that of the reported largest β-glucosidase (240 kDa; Watanabe et al., 1992) purified from *A. niger* (although both enzymes have similar molecular weight of mono-

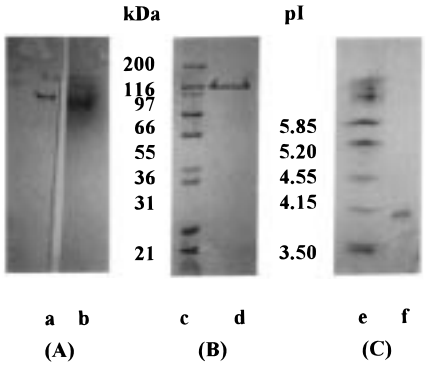

Figure 2. PAGE of the purified *A. niger* β-Glu II: (A) native-PAGE [lane a, stained with Coomassie Brilliant Blue; lane b, activity stained with esculin (~6 μg of the purified enzyme was applied to each lane)]; (B) SDS−PAGE (lane c, marker proteins; lane d, purified β-Glu II (~6 μg of sample was applied)]; (C) IEF−PAGE [lane e, marker proteins; lane f, purified β-Glu II (~6 μg of sample was applied)].

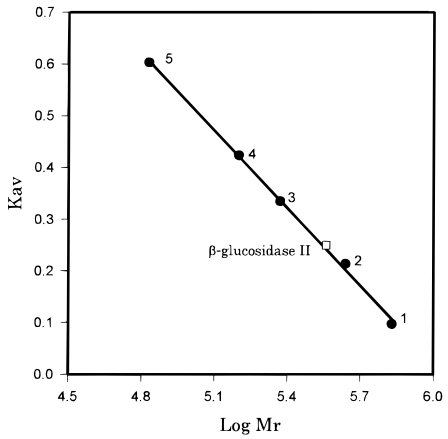

Figure 3. Estimation of molecular mass of *A. niger* β-Glu II by Sephacryl S-300 gel filtration. The molecular mass of the native form of the β-Glu II (□) was determined by Sephacryl S-300 gel filtration and calibrated with the following standard proteins (●): 1, thyroglobulin (669 kDa); 2, ferritin (440 kDa); 3, catalase (232 kDa); 4, aldolase (158 kDa); and 5, bovine serum albumin (67 kDa).

Table 2. Effects of Organic Solvents on *A. niger* β-Glu II

solvent	relative activity (%) at solvent concn of				
	0% v/v	10% v/v	20% v/v	30% v/v	40% v/v
methanol	100	174	180	72	84
ethanol	100	143	151	145	109
1-propanol	100	135	139	125	82
2-propanol	100	107	112	105	111
1-butanol	100	140	123	106	105
2-butanol	100	121	125	130	71
cyclohexanol	100	127	112	112	120
acetone	100	100	89	72	50
acetonitrile	100	86	83	67	0
dimethyl sulfoxide	100	98	64	12	0
N,*N*-dimethylformamide	100	110	90	53	20

[a] The activity measured without any organic solvent addition was considered 100%.

mer) and is similar to that of the β-glucosidase (Glu I) purified from *Botrytis cinerea* (Gueguen et al., 1995). However the *B. cinerea* Glu I is composed of four subunits.

A homology search, utilizing the computer program BLASTN (Altschul et al., 1990), revealed a significant degree of identity (94−50%) between the 19 N-terminal amino acid sequence and numerous β-glucosidases

Table 3. Substrate Specificity of *A. niger* β-Glu II

substrate	specific activity (units/mg of protein)	relative activity (%)
p-nitrophenyl β-D-glucoside	366[a]	100
cellobiose	464[b]	100
salicin	58[b]	12.5
phloridizin	0[c]	0
arbulin	26[b]	5.7
esculin	42[b]	9

[a] Specific activity is reported as μmol of *p*-nitrophenol released min^{-1} mg^{-1}. [b] Specific activity is reported as μmol of glucose equivalent released min^{-1} mg^{-1}.

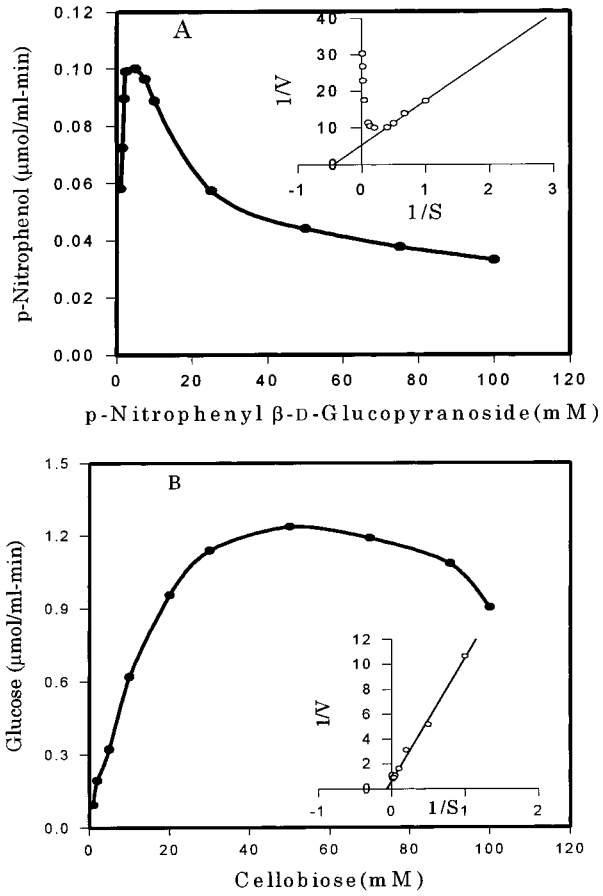

Figure 4. Effect of various concentrations of pNPG (A) and cellobiose (B) on the activity of purified β-Glu II from *A. niger*. (Inset) Lineweaver−Burk plot.

(including some proteins without β-glucosidase activity) from different sources. Among these proteins, the β-glucosidases of *A. niger* (Novo SP188) (Himmel et al., 1993), *A. aculeatus* (Kawaguchi et al., 1996), and *A. kawachii* (unpublished data) were found to have higher homology than other proteins (94%, 94%, and 83% identity, respectively).

The β-Glu II was completely inhibited by Fe^{2+} at 5.0 mM, and half the activity of the enzyme was inhibited by Ag^{+} and Fe^{3+} at 5.0 mM. It has been demonstrated that the enzyme from *A. ornatus* (Pemberton et al., 1980) is also inhibited by Ag^{+} and Fe^{2+}. However, the enzyme from *A. terreus* (Workman and Day, 1982) was relatively unaffected by metal ions, and the enzyme from *A. japonicus* (Sanyal et al., 1988) was also unaffected by metal ions except for Hg^{2+}. On the contrary, the activity of β-glucosidase from *Streptomyces reticuli* was stimulated by $MgCl_2$ (50 mM) or $CaCl_2$ (6 mM) at 1.5 and 2.0-fold, respectively (Huepel et al., 1993). The

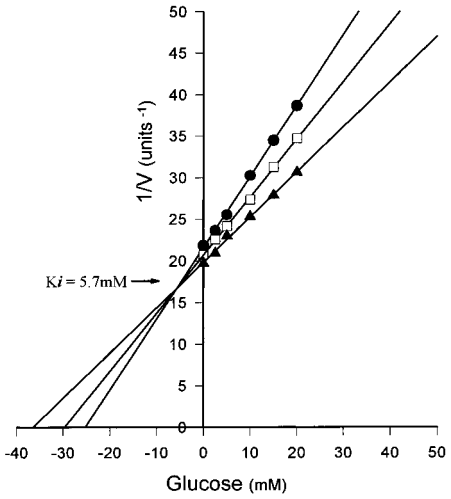

Figure 5. Dixon plot of inhibitory effects of glucose on pNPG hydrolysis by *A. niger* β-Glu II. The reaction time was 10 min at pH 4.5 and 50 °C. The enzyme was used at 1.0 unit/mL. The pNPG concentrations used were 5.0 mM (●), 6.0 mM (□), and 8.0 mM (▲).

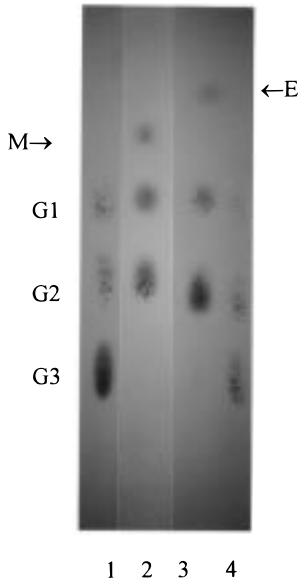

Figure 6. TLC chromatograms of the transglycosylation products produced by *A. niger* β-Glu II in different reaction systems: lanes 1 and 4, standards (G1, glucose; G2, cellobiose; G3, cellotriose); lane 2, products from 20% methanol; lane 3, products from 20% ethanol; spot M, methyl glucoside; spot E, ethyl glucoside.

chelating agents EDTA and *o*-phenanthroline did not affect the enzyme activity, indicating that divalent cations are not essentially required for enzyme activation. Addition of dithiothreitol, 2-mercaptoethanol, cysteine, iodoacetic acid, and phenylmethanesulfonyl fluoride did not result in a significant increase or decrease in activity, suggesting that the thiol group and hydroxyl group are not essential for the enzyme catalytic center. The enzyme from *A. japonicus* (Sanyal et al., 1988) was unaffected by EDTA and *N*-ethylmaleimide but was extremely sensitive to *p*-chloromercuric benzoate. The enzyme from *Pyrococcus furiosus* (Kengen et al., 1993) was unaffected by the presence of dithiothreitol, 2-mercaptothanol, iodoacetamide, and *N*-ethylmaleimide.

In a general, β-glucosidases can preferentially utilize alcohols rather than water as acceptors for the glucosyl moiety during catalysis of pNPG, resulting in elevated reaction rate (Premberton et al., 1980; Saha et al., 1994). The β-Glu II is stable and activated in limited concentrations of alcohol. Its activity was higher in primary vs secondary alcohols. This indicates that primary alcohols provided not only better hydrolysis conditions for the enzyme, but also provided themselves as better acceptors for the β-glucosyl residues. At higher concentrations, the enzyme was inhibited by those alcohols, probably because of protein denaturation.

The purified β-Glu II has a high substrate specificity. It can tolerate a wide variety of aglycons, provided that the glycosyl residue of the substrate has the β-D-glucosyl configuration. A number of β-glucosidases from various biological sources are accompanied by β-galactosidase and/or β-xylosidase activity (Kengen et al., 1993; McCleary and Harrington, 1988; Sanyal et al., 1988; Woodward and Wiseman, 1982; Yeoh et al., 1988; Yan and Lin, 1997). In contrast, our purified β-Glu II showed absolutely no activities toward lactose, *p*-nitrophenyl β-xyloside, *p*-nitrophenyl β-galactoside, and *p*-nitrophenyl β-mannoside. These results indicated that the β-Glu II recognizes strictly the C-4 configuration of the terminal, nonreducing β-D-glucose residue in the substrate. The β-glucosidases purified by Unno et al. (1993) and Watanabe et al. (1992) also had high substrate specificity toward β-glucosidic linkages. However, these two enzymes showed broader substrate specificity than the β-Glu II.

A broad range of K_m values for pNPG and cellobiose has been reported from different fungal sources, including *Aspergillus* species (Christakopoulos et al., 1994; Gueguen et al., 1995; Kengen et al., 1993; Sanyal et al., 1988; Watanabe et al., 1992; Woodward and Wiseman, 1982; Workman and Day, 1982; Yeoh et al., 1986). These may be attributed to different conditions employed for its determination. The kinetic properties of the purified β-Glu II revealed that the enzyme had a higher affinity toward pNPG than cellobiose, but the maximum reaction rate of the enzyme toward cellobiose was higher than that of the pNPG. The turnover rate (k_{cat}) of cellobiose was ~3-fold that of pNPG. Therefore, the β-Glu II is considered as a cellobiase with aryl-β-glucosidase activity. The enzyme has a significant substrate inhibition with different substrates. It was observed that at pNPG concentration >7.5 mM and at the concentration of cellobiose >50 mM, there was a strong inhibition of the enzyme activity (Figure 4). Substrate inhibition of β-glucosidase has also been reported from different sources (Christakopoulos et al., 1994; Saha et al., 1994; Yeoh et al., 1986).

The inhibition by glucose, which is a common characteristic of β-glucosidases (Saha et al., 1994; Woodward and Wiseman, 1982; Yeoh et al., 1986), although there are some exceptions (Christakopoulos et al., 1994; Saha and Bothast, 1996), is an important constraint for industrial use of these enzymes. Most of the microbial enzymes were competitively inhibited by glucose with K_i values in the range of 0.6−8 mM. The purified β-Glu II was competitively inhibited by glucose at a K_i of 5.7 mM, which is similar to that of *A. ornatus* (Yeoh et al., 1986) and *Aureobasidium pullulans* (Saha et al., 1994). This indicated that the purified enzyme, like other β-glucosidases (Watanabe et al., 1992; Kengen et al., 1993), has glucosyltransferase activity as well as hydrolysis activity. When the purified β-Glu II was reacted with cellobiose under neutral conditions, forma-

tion of cellotriose was obtained in a short reaction time, and in the presence of alcohols, formation of alkyl glucosides was observed. β-Glucosidase with high glucosyltransferase activity makes the enzyme more valuable in the biotechnological industry for production of some oligosaccharides with special functionality as healthy food additives; alkyl glucosides can be applied as nonionic surfactants in detergents, foods, and pharmaceuticals.

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