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Characterization of Novel β-Glucosidases with Transglycosylation Properties from *Trichosporon asahii*

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ABSTRACT: Two novel β-glucosidases from *Trichosporon asahii*, named BG1 and BG2, were purified to electrophoretic homogeneity using ammonium sulfate precipitation, hydrophobic interaction, ion exchange, and gelfiltration chromatography. The molecular weight of BG1 and BG2 were estimated as 160 kDa and 30 kDa, respectively. The K_m, V_{max}, K_{cat}, and K_{cat}/K_m values of the two β-glucosidases for *p*-nitrophenyl-β-D-glucopyranoside were determined. Both enzymes showed relatively high affinity to *p*-nitrophenyl-β-D-glucopyranoside in 4-nitrophenol glycosides and gentiobiose in saccharide substrates. The enzymes exhibited optimum activity at pH 6.0 and pH 5.5, respectively. Their respective optimum temperatures were 70 and 50 °C. Metal ions and inhibitors had different effects on the enzymes activities. Circular dichroism (CD) spectroscopy demonstrated that the purified BG1 exhibited a β-sheet-rich structure and that BG2 displayed a high random coil conformation. HPLC analysis of transglycosylation and reverse hydrolysis assays revealed that only BG1 possessed transglycosylation activity and synthesized cello-oligosaccharides by the addition of glucose. This suggested that BG1 could be used to produce complex bioactive glycosides and could be considered as a potential enzyme for industrial application.

KEYWORDS: β-glucosidase, *Trichosporon asahii*, purification, circular dichroism, transglycosylation

INTRODUCTION

β-Glucosidases (β-D-glucoside glucohydrolase; EC 3.2.1.21) constitute a group of well characterized, biologically and industrially important enzymes.[1] β-Glucosidases normally catalyze the hydrolysis of the β-1,4-glycosidic bond present in short-chain oligosaccharides (containing 2−6 monosaccharides), liberating the aglycones, such as norisoprenoids, aliphatic alcohols, sesquiterpenes, resveratrol, monoterpenes, volatile phenols, and other benzyl derivatives as found in many fruits or juices.[2]

As a representative of a heterogeneous group, β-glucosidases have been suggested for many industrial applications, such as flavor enhancement (wine, fruit juice, and tea), and the production of aromatic products, biodegradable nonionic surfactants, and other compounds.[3,4] Coupled with endoglucanases (1,4-β-D-glucano-hydrolase; EC 3.2.1.4) and exocellobiohydrolase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.91), β-glucosidases are also involved in complete enzymatic degradation of cellulosic substrates.[5] In addition, β-glucosidases are thought to be directly involved in cellulase induction.[6] Variations of β-glucosidases are widely distributed in all types of living organisms, including different plants and fruits, and play important roles in diverse biological processes.[1,7] β-Glucosidases are also important enzymes for carbohydrate metabolism in bacteria, archaea, and eukarya. Therefore, the enzymes can be produced by bacteria, yeast, and fungi.[7,8]

Although the use of enzymes in industrial processes is becoming increasingly widespread for catalytic efficiency, some great challenges are emerging both in seeking an appropriate, safe, and alternative method for classical chemical reactions and in finding applications for some enzymes with desirable activities and properties in industry.[9,10] Transglycosylation is a particularly useful reaction in industry. Enzyme-catalyzed synthesis allows the formation of well-defined oligosaccharides selectively in the absence of any protecting groups. A large number of well-characterized glycosyltransferases catalyze the transfer of sugars from nucleoside-diphosphate intermediates to mono-, oligo-, and polysaccharide acceptor moieties.[1,9] Thus, these enzymes have been used for the synthesis of alkyl glycosides, synthetic flavor precursors, precursors of glycolipids, glycosylated oligopeptides, and oligosaccharides.[4,11] β-Glucosidases not only catalyze the hydrolysis of glycosidic bonds but also can be used for glycoside formation. Compared with those of glycosyltransferases, easy availability and inexpensiveness make β-glucosidases especially attractive for oligosaccharide synthesis. Such deep interests created in enzymes make glucosidases useful in the synthesis of diverse oligosaccharides, especially those glucosidases possessing transglycosylation properties, which are in high demand for the production of bioactive compounds.[10]

In this work, we report the purification, characterization, and transglycosylation properties of β-glucosidases from a newly isolated yeast, *Trichosporon asahii*, which was confirmed as a good source of β-glucosidase in our previous study.[12] Furthermore, an estimation of the secondary structures of β-glucosidases in solution was made from the CD spectra. To our knowledge, this is the first study with the aim to investigate the characterization and transglycosylation properties of extracellular β-glucosidases from a *T. asahii* strain.

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MATERIALS AND METHODS

Chemicals and Reagents. 4-Nitrophenyl-β-D-glucopyranoside (pNPG), 4-nitrophenyl-β-D-fucopyranoside (pNPF), 4-nitrophenyl-β-D-galactopyranoside (pNPGL), sophorose, laminaribiose, gentiobiose, trehalose, maltose, maltotriose, maltopentaose, cellobiose, cellotriose, cellotetraose, and high performance liquid chromatography (HPLC)-grade solvents were purchased from Sigma Chemical Co. (St. Louis, MO). SDS (sodium dodecyl sulfate), tris, glycine, β-mercaptoethanol, Coomassie brilliant blue R-250, acrylamide/bis acrylamide, and glucose oxidase/peroxidase reagent were obtained from Genei (Bangalore, India). Phenyl-Sepharose Q FF column, Sephacryl S-300 (16/60) column, and protein molecular weight markers were purchased from Amersham Biosciences (Uppsala, Sweden). Dialysis membranes and ultrafiltration membranes were purchased from Millipore, USA. All other chemicals were of reagent grade or chromatographic grade.

Medium Preparation and Enzyme Localization Studies. A β-glucosidase high-producing yeast, *T. asahii*, isolated from vineyard soil, was cultured in an optimized liquid synthetic medium containing (%, w/v) dextrin 4.67%, yeast extract 2.99%, $MgSO_4$ 0.01%, and K_2HPO_4 0.02%.[12] The medium was autoclaved at 121 °C for 20 min. Each flask was inoculated with 5% (v/v) seed culture prepared earlier and incubated at 28 °C, 200 rpm for 72 h. A protocol according to Ferreira was followed to determine the cellular localization of the enzyme.[13]

Enzyme Activity and Protein Assay. β-Glucosidase activity assay was performed by measuring the amount of *p*-nitrophenol (pNP) released from a synthetic substrate generally used for glucosidase assays, pNPG in a 96-well microtiter format based method with the following modifications.[14] The 5 μL of enzyme solution was mixed with 0.1 mL of 5 mM pNPG solution in 0.1 M citrate phosphate buffer at pH 5.0. The reaction mixture was incubated at 50 °C for 30 min, and the enzymatic reaction was subsequently stopped by adding 0.2 mL of 1.0 M Na_2CO_3. The pNP released from this mixture was measured in a microplate reader (Multiskan spectrum, Thermo Scientific Inc.) at 400 nm. A unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of pNP per minute under the assay conditions. Protein was assayed using Coomassie blue (Bradford) protein assay reagent according to the technical instruction manual, with BSA as the standard.

Purification of β-Glucosidase. All purification steps were carried out at 4 °C unless otherwise specified. The purifications of BG1 and BG2 from *T. asahii* were done following the same procedure.

Ammonium Sulfate Precipitation. Culture broth was centrifuged at 8,000*g* for 20 min to remove the cells. Proteins were precipitated by the addition of ammonium sulfate (up to 80% saturation) to culture the supernatant. The mixture was centrifuged at 14,000*g* for 30 min. Then, the precipitate was dissolved in 20 mL of 50 mM Tris-HCl buffer (pH 8.0) and dialyzed overnight against the same buffer containing 1.4 M ammonium sulfate with three changes of the buffer.

Hydrophobic Interaction Chromatography. The dialyzed enzyme solution was loaded onto the HiPrep16/10 phenyl FF (high sub) Phenyl-Sepharose column, previously equilibrated with buffer A (50 mM Tris-HCl, pH 8.0, and 1.4 M ammonium sulfate). Elution of the β-glucosidase was performed by decreasing the concentration of $(NH_4)_2SO_4$ from 1.4 to 0 M at a flow rate of 5 mL/min with a step gradient in 100 mL of buffer A and 100 mL of buffer B (50 mM Tris-HCl, pH 8.0). The active fractions were pooled and concentrated using an Amicon YM-10 membrane fitted into a 50 mL stirred ultrafiltration cell with a cutoff of 10 kDa.

Ion Exchange Chromatography. The concentrated fractions pooled from the previous step were dialyzed against buffer B overnight with two changes of the buffer and applied onto a 5 mL HiTrap Q FF column equilibrated with buffer B. The β-glucosidase was eluted using a step gradient in 100 mL of buffer B and 100 mL of buffer C (50 mM Tris-HCl, pH 8.0 containing 1.0 M NaCl). The flow rate was set at 5 mL/min, and 1.5 mL fractions were collected. Fractions with β-glucosidase activity were pooled and concentrated by ultrafiltration through a membrane with a cutoff of 10 kDa.

Gel Filtration Chromatography. The pooled fractions were subjected to gel filtration chromatography on a HiPrep Sephacryl S-300 (16/60) High Resolution column equilibrated with 50 mM Tris-HCl, pH 8.0, and 0.15 M NaCl. The flow rate was set at 0.5 mL/min and the pressure kept below 0.15 MPa. Fractions with β-glucosidase activity eluted from the gel filtration column were pooled and concentrated by ultrafiltration. Enzyme activity and protein concentration of pooled fractions were determined. The specific activity of the enzyme was calculated after each step of purification.

Electrophoretic Analyses. Samples were analyzed on SDS−PAGE (12.5% separating and 4.5% stacking) according to Laemmli to check the purity and determine the molecular weight of the purified enzyme.[15]

Kinetic Parameters. To determine the kinetic parameters (K_m, V_{max}, and k_{cat}/K_m), the purified enzymes were assayed at substrate (pNPG) concentrations ranging from 0.01 to 20 mM under standard assay conditions. The enzymes were prepared at a concentration of 30 μg/mL in 0.1 M citrate phosphate buffer (pH 5.0). Kinetic parameters (K_m and V_{max}) were determined by the Michaelis−Menten and Hanes−Woof plots. The specificity constants, k_{cat}/K_m, were calculated to determine the substrate specificity of each enzyme.

pH and Temperature Optima and Stability. The following buffers (50 mM) were used for the pH assays: glycine-HCl (pH 2.0−5.0), sodium acetate (pH 5.0−6.0), sodium phosphate (pH 6.0−8.0), Tris-HCl (pH 8.0−9.0), and glycine-NaOH (pH 9.0−11.0). To determine the optimum pH, the activity of β-glucosidases was assayed in the buffers of different pH values (2.0−11.0) at 50 °C. In order to investigate the pH stability, β-glucosidase fractions were incubated at various pHs (2.0−11.0) at 4 °C for 1 h, and the residual activity was measured using pNPG as the substrate according to the standard method. The effect of temperature on the activity of β-glucosidases was studied by carrying out enzyme assays in 0.1 M citrate phosphate buffer at different temperatures (20−90 °C) and at pH 5.0. Thermostability of the purified enzymes was measured by incubating the β-glucosidase alone at different temperatures (20−90 °C) for 1 h. After cooling of the treated enzymes on ice for 30 min, the residual β-glucosidase activities were measured according to the standard assay method.

Effect of Metal Ions and Chemical Reagents. The purified β-glucosidases were incubated with various metal ions (20 mM) and reagents in 0.1 M citrate phosphate buffer (pH 5.0) for 30 min at room temperature. Activities were then measured using pNPG as the substrate under standard assay conditions in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was taken as 100%. The results presented were the average of three trials.

Substrate Specificity. Substrate specificities of the purified enzymes were determined by incubating the purified enzyme with respective substrates (5 mM) in 0.1 M citrate phosphate buffer at 50 °C for 30 min and measuring the liberated pNP or glucose by the standard procedures.[16] Relative activities were calculated as a percentage of substrate pNPG.[3]

Circular Dichroism Spectra Epsilons. Circular dichroism (CD) analysis was conducted at room temperature with a spectropolarimeter (French Biologic Company, Grenoble, French; Model MOS-450). The far-UV spectrum of β-glucosidases from *T. asahii* were measured over a wavelength range of 190−240 nm as an average of five spectra with 100 nm/min scan speed and a step resolution of 0.1 nm. All measurements were made in a 0.1 cm path length cuvette using protein at a concentration of 30 μg/mL (10 mM phosphate buffer, pH 5.0). All spectra were corrected by subtracting blank runs on protein free buffer solutions. Analysis of the protein secondary structure was performed using Dichroweb.[17] The CD data were expressed in terms of mean residue ellipticity, $[\theta]$, in deg cm^2 /dmol.

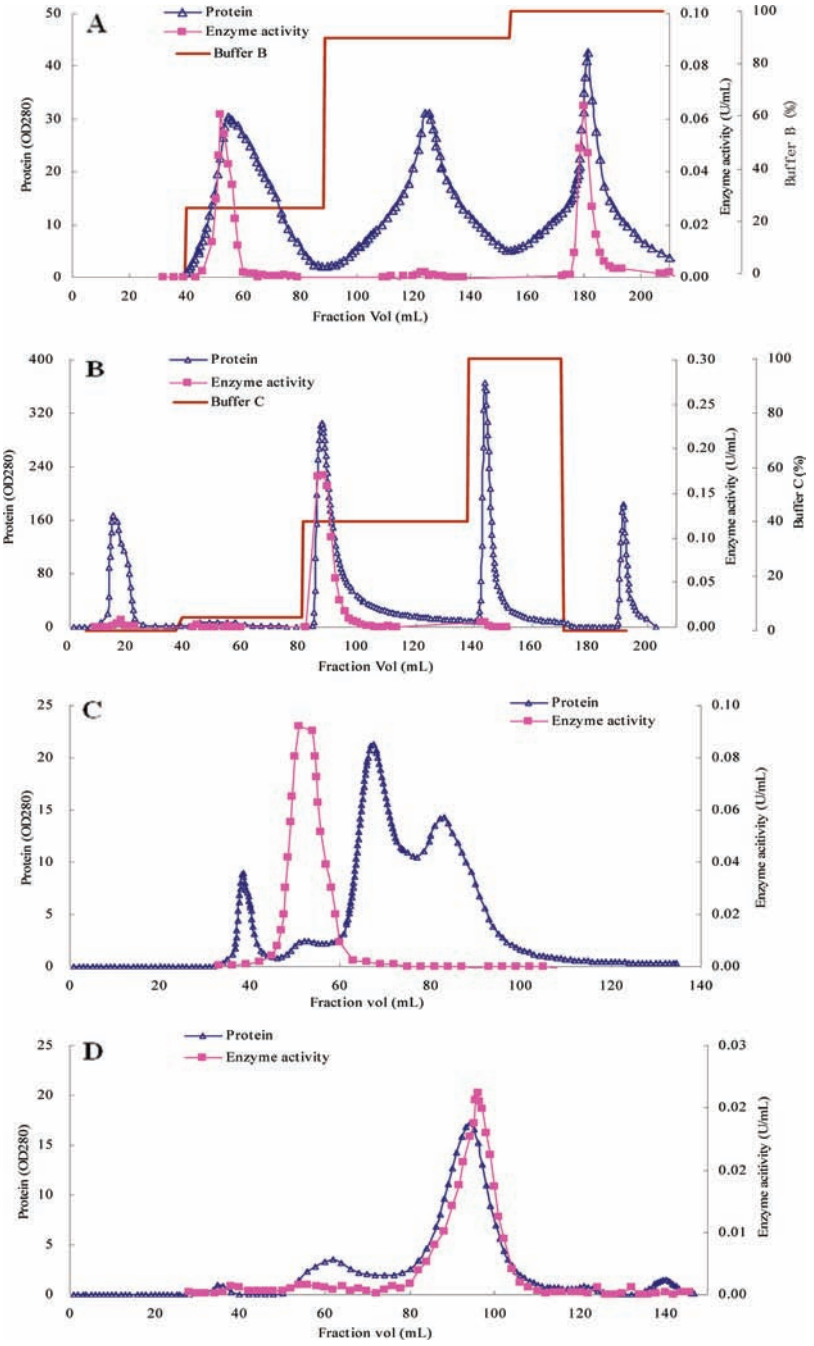

Figure 1. Purification of β-glucosidases. The enzymes were purified through (A) Phenyl-Sepharose HiPrep16/10 (the first active fraction was BG1, and the latter was BG2), (B) Q FF ion exchange chromatography, (C) Sephacryl S-300 gel chromatography (BG1), and (D) Sephacryl S-300 gel chromatography (BG2).

Transglycosylation Assay. Reverse hydrolysis and transglycosylation catalyzed by β-glucosidases were carried out with anhydrous glucose and cellobiose as substrates according to the method of Pal with the following modifications.[9] Purified enzymes with activity unit 1 U/mL and 1.5 g of anhydrous glucose or 1.0 g of cellobiose substrates were incubated in 2 mL of citrate-phosphate buffer (0.1 M, pH 5.0) at 50 °C. Aliquots (0.5 mL) (4 h and 8 h for glucose; 6 h and 12 h for cellobiose) were mixed with 4 volumes of 100% ethanol prefreezed at −20 °C for 1 h. Then, samples were centrifuged at 14,000*g* for 20 min to remove protein. The supernatant was concentrated under a nitrogen flow to 500 μL and analyzed for the content of sugars by the HPLC method. The oligosaccharide products were detected using a Sugar-Pak TMI column (6.5 × 300 mm; Waters Corporation, MS, USA) with double distilled water as the mobile phase. Individual sugar concentrations were determined by calculating the area of their peaks in relation to the total peak area. A control experiment without any enzymes was performed under the same conditions.

RESULTS AND DISCUSSION

Location of *T. asahii* β-Glucosidase. The β-glucosidase activity of *T. asahii* was determined in whole cells (parietal

Table 1. Summary of the Purification Profile of β-Glucosidases

purification step		total protein (mg)	specific activity (U/mg)	activity (U)	purification fold	yield (%)
crude		280	0.159	44.6	1	100
precipitation		145	0.223	32.3	1.40	72.5
Phenyl-Sepharose	BG1	11.9	0.546	6.47	3.40	14.5
	BG2	10.4	0.692	7.19	4.32	16.1
Q FF	BG1	1.37	1.94	2.66	12.2	5.96
	BG2	1.07	2.58	2.77	16.2	6.20
Sephacryl S-300	BG1	0.31	3.59	1.11	22.5	2.49
	BG2	0.34	4.59	1.56	28.8	3.50

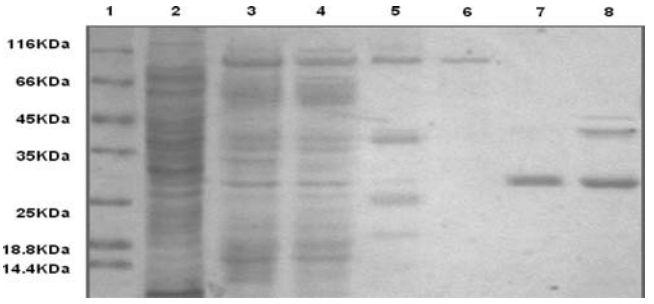

Figure 2. SDS−PAGE analysis of purified β-glucosidases. Lane 1, protein marker; lane 2, ammonium sulfate precipitation; lane 3, Phenyl-Sepharose (BG2); lane 4, Phenyl-Sepharose (BG1); lane 5, Q FF (BG1); lane 6, Sephacryl S-300 (BG1); lane 7, Sephacryl S-300 (BG2); lane 8, Q FF (BG2).

activity), in permeabilized cells (intracellular activity), and in the medium (extracellular activity). The final total β-glucosidase activity was 0.52 U/mL. On average (n = 3), the extracellular fraction (supernatant) contained the majority of the enzyme activity (40.7%) followed by the permeabilized fraction (intracellular) (36.5%), and the lowest whole cell (parietal) activity (22.8%) was detected. β-Glucosidase activity can exist in the supernatant, whole cell, cell wall, cytosol, and membrane. The location of β-glucosidase activity is strain dependent.[18] The investigation of the enzyme activity location indicated that β-glucosidase from *T. asahii* appeared to be chiefly secreted into the medium.

Purification of Extracellular β-Glucosidase. The β-glucosidase activity in the culture supernatant was 0.21 U/mL. The enzyme was purified successively through ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, and gel filtration according to the procedures described in the Materials and Methods section. The enzyme was fractionated into two pools containing β-glucosidase activity in Phenyl-Sepharose fast flow (named BG1 and BG2). BG1 was eluted by buffer B containing 1.05 M ammonium sulfate, and BG2 was eluted by buffer B (no ammonium sulfate) (Figure 1A).

The two active fractions issued from the previous step were further purified on a Q FF column (Figure 1B), respectively. Both fractions were all washed out with buffer C containing 0.4 M NaCl. The enzymes were finally purified to homogeneity by a HiPrep Sephacryl S-300 (16/60) High Resolution gel filtration column (Figure 1C, D) on an AKTA Explorer system. The results of the purification procedure are summarized in Table 1. During the purification process for BG1, a recovery of 2.49% total activity with a 22.5-fold purification was obtained, while BG2 was

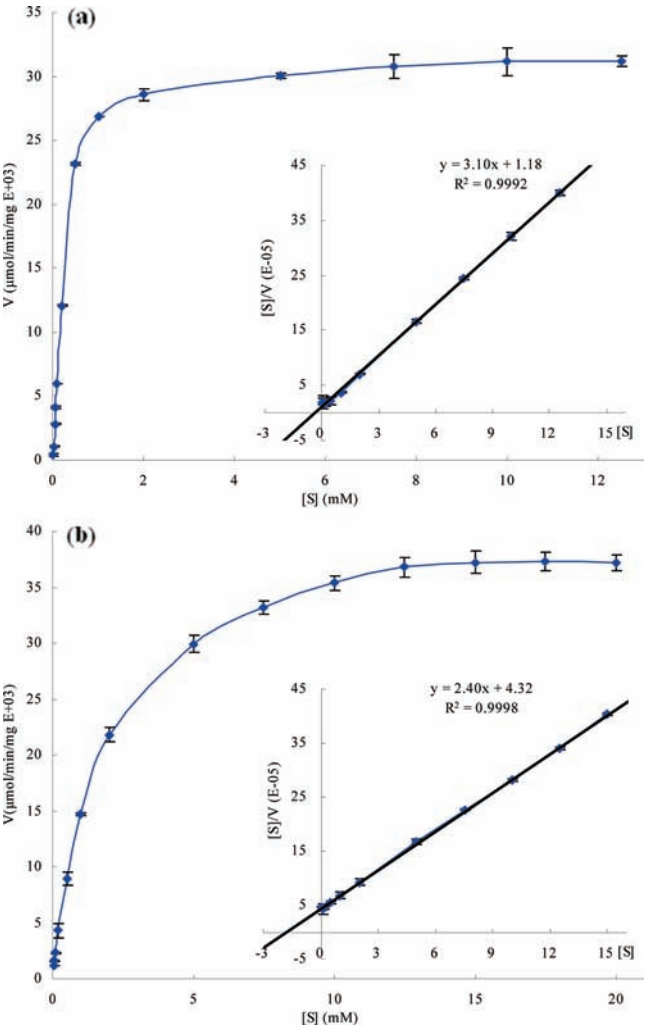

Figure 3. Michaelis−Menten and Hanes−Woof plots for BG1 (a) and BG2 (b) activities.

purified to 28.8-fold with a specific activity of 4.59 U/mg and an overall yield of 3.50%. The purified enzymes all showed a single band on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (Figure 2).

The purity and molecular weight were estimated by both gel filtration chromatography and SDS−PAGE. The molecular weight of BG1 was estimated to be 80 kDa by SDS−PAGE and 160 kDa by gel filtration chromatography, respectively. However, the molecular weight of BG2 was estimated about

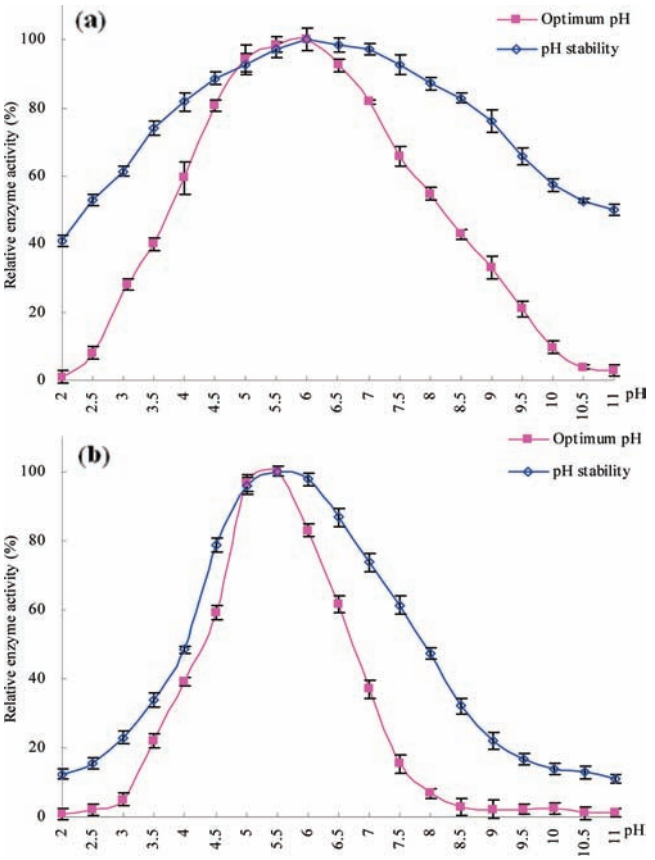

Figure 4. Effects of pH on the activities of BG1 (a) and BG2 (b). The tests were performed at 50 °C using 5 mM pNPG in 50 mM different buffers (glycine-HCl, pH 2.0−5.0; sodium acetate, pH 5.0−6.0; sodium phosphate, pH 6.0−8.0; Tris-HCl, pH 8.0−9.0; and glycine-NaOH, pH 9.0−11.0) set to the appropriate pH and incubated for 30 min (for optimum pH) or 1 h (for pH stability).

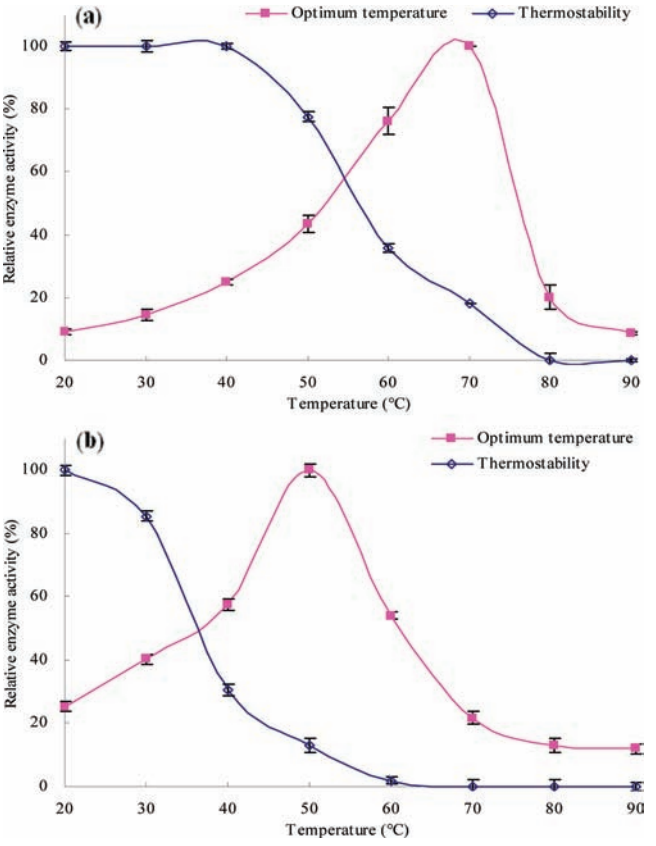

Figure 5. Effects of temperature on the activities of BG1 (a) and BG2 (b). The tests were conducted using 5 mM pNPG in 100 mM citrate phosphate buffer (pH 5.0) and incubation for 30 min (for optimum temperature) or 1 h (for thermostability) at different temperatures.

30 kDa by both SDS−PAGE and gel filtration chromatography. The enzymes exhibiting subunit molecular weight smaller than 70 kDa are generally monomeric, whereas large subunit enzymes (70−120 kDa) are observed to be multimeric.[19,24,25] As SDS−PAGE and gel filtration reflect the denatured and native state, respectively, the data above suggests that the native BG1 may consist of two identical subunits and that the native BG2 may be a monomeric enzyme. It has been reported by other research groups that β-glucosidases purified from different microorganisms possessed molecular weights ranging from several tens of thousands to several hundreds of thousands daltons.[8,12,26] The high molecular weight of the β-glucosidase BG1 is in agreement with those of many extracellular β-glucosidases characterized from other fungal sources.[14,19]

Kinetic Parameters. Kinetic parameters were determined using different pNPG concentrations. The purified enzymes obeyed the Michaelis−Menten equations over the substrate concentration range (Figure 3). The K_m and V_{max} values of BG1 were calculated by the Hanes−Woof method to be 0.382 mM and 3.23 × 10^4 μmol/min/mg, respectively. The K_m and V_{max} of BG2 were determined as 1.80 mM and 4.17 × 10^4 μmol/min/mg. According to Sutheera Khantaphant, the values of $K_{cat_{BG1}}$ (8.61 × 10^4 s^{-1}), $K_{cat_{BG2}}$ (2.08 × 10^4 s^{-1}), $K_{cat}/K_{m_{BG1}}$ (2.25 × 10^5 $s^{-1}mM^{-1}$), and $K_{cat}/K_{m_{BG2}}$ (1.16 × 10^4 $s^{-1}mM^{-1}$) were obtained.[20,21] The results showed that BG1 had more affinity for pNPG and higher catalytic efficiency than BG2 ($K_{m_{BG1}} < K_{m_{BG2}}$, $K_{cat_{BG1}} > K_{cat_{BG2}}$, and $K_{cat}/K_{m_{BG1}} > K_{cat}/K_{m_{BG2}}$).

pH and Temperature Optima and Stability. The effect of pH on β-glucosidase activity was examined over a pH range of 2.0−11.0 at 50 °C using pNPG as the substrate (Figure 4). BG1 was active at a pH range of 3.0 to 9.5, and the enzyme showed its highest activity at pH 6.0. However, BG2 was only active in a narrower pH range (3.5−7.5), and its activity was optimal at pH 5.5. The optimum pHs of BG1 and BG2 were similar to those of most other β-glucosidases (pH range 4−6).[5]

The pH stability test revealed that BG1 was fairly stable in a pH range of 4.0 to 8.5, with 81.8% and 82.9% of the initial activity remaining at these pHs. In comparison, only 48.3% and 32.2% of the original activity was retained at the same pHs for purified BG2 after 1 h of incubation. Although both enzymes were all denaturalized by extreme acidic pH, BG1 exhibited higher specific activity (61.2% residual activity) than BG2 (22.9% residual activity) at pH 3.0. These results indicated that BG1 was more active and stable in a wider range of pH values than BG2.

The effect of temperature on β-glucosidase activity was determined by assaying enzyme activity at different temperatures (20−90 °C). BG1 and BG2 exhibited maximum activity at 70 and 50 °C, respectively (Figure 5). In addition, BG1 showed 76.2% and 20.3% of its maximum activity at 60 and 80 °C, respectively, while BG2 displayed approximately over 50% of its maximal activity when tested at 40 and 60 °C. Many researchers reveal that β-glucosidases from different microorganisms possess

Table 2. Susceptibility of β-Glucosidases toward Metal Ions and Inhibitors

compounds	concentrations	residual activity (%)[a] BG1	BG2
none		100	100
Li^+ (Li_2SO_4)	20 mM	86.94 ± 2.59	74.04 ± 1.95
Zn^{2+} ($ZnSO_4 \cdot 7H_2O$)	20 mM	79.31 ± 3.66	87.13 ± 1.59
Mn^{2+} ($MnSO_4 \cdot H_2O$)	20 mM	94.58 ± 1.82	76.49 ± 1.89
Ni^{2+} ($NiSO_4 \cdot 6H_2O$)	20 mM	31.10 ± 4.53	33.67 ± 4.71
Fe^{2+} ($FeSO_4 \cdot 7H_2O$)	20 mM	117.48 ± 7.16	130.58 ± 1.78
Co^{2+} ($CoCl_2 \cdot 2H_2O$)	20 mM	53.52 ± 4.14	73.18 ± 3.90
Mg^{2+} ($MgSO_4$)	20 mM	98.27 ± 4.40	58.63 ± 2.22
K^+ (KCl)	20 mM	86.40 ± 1.36	82.44 ± 3.34
Ca^{2+} ($CaCl_2$)	20 mM	86.23 ± 3.82	48.17 ± 1.95
Fe^{3+} ($FeCl_3$)	20 mM	87.76 ± 9.57	45.49 ± 0.65
Ag^+ ($AgNO_3$)	20 mM	23.43 ± 2.65	28.61 ± 2.90
Pb^{2+} ($C_4H_6O_4Pb \cdot 3H_2O$)	20 mM	57.66 ± 8.57	78.16 ± 1.91
Al^{3+} ($AlCl_3$)	20 mM	94.97 ± 5.51	80.41 ± 1.27
Ba^{2+} ($BaCl_2$)	20 mM	85.06 ± 2.38	61.27 ± 3.20
Cu^{2+} ($CuSO_4 \cdot 5H_2O$)	20 mM	98.69 ± 2.47	77.21 ± 2.41
methanol	10%	106.86 ± 1.18	96.29 ± 1.23
ethanol	10%	96.49 ± 2.55	96.37 ± 1.82
isopropanol	10%	106.25 ± 6.45	93.94 ± 1.89
SDS	1%	108.32 ± 5.99	20.34 ± 9.82
β-mercaptoethanol	1%	115.67 ± 6.94	87.68 ± 3.34
Triton X-100	1%	96.94 ± 2.31	90.59 ± 2.00
EDTA	1%	43.01 ± 2.62	7.46 ± 0.76

[a] Values are the means ± SD (n = 3).

optimum temperatures ranging from 20−80 °C and seem to be strain dependent.[19,22] The results suggest that the different β-glucosidases from the same strain also could exhibit different optimum pH or temperature, which coincided with some literature.[5,23]

Thermostability was examined by measuring the residual activity after 1 h of incubation at different temperatures. The purified BG1 was highly stable below 40 °C, but the activity sharply decreased when the temperature was above 60 °C. The enzyme activity was almost completely lost at 80 °C. In contrast, BG2 was more sensitive to temperature and only stable at 20 °C. BG2 lost almost 15% and 70% of its maximum activity at 30 and 40 °C, respectively, after a 1 h exposure. This suggests BG1 has better thermal stability than BG2.

Susceptibility toward Metal Ions and Chemical Reagents. The purified enzymes were incubated in 0.1 M citrate phosphate buffer (pH 5.0) containing various metal ions and inhibitors, then the residual activities were determined under standard enzyme assay conditions. The effects of metal ions and inhibitors on β-glucosidase activity are shown in Table 2. After 30 min of incubation with Ni^{2+}, Co^{2+}, Ag^+, and Pb^{2+}, only 23.4% to 57.7% and 28.6% to 78.2% relative activities remained for BG1 and BG2, respectively. The susceptibility to Ag^+ and Co^{2+} suggest that a sulfhydryl group may be involved in the active catalytic site of BG1 and BG2.[5,24]

Less inhibitory effects on the activities of BG1 and BG2 were observed in the presence of Li^+, Zn^{2+}, Mn^{2+}, and K^+. Compared with that of BG2 and β-glucosidase from fungi *Periconia sp.*, purified BG1 was also less susceptible to the cations Mg^{2+}, Ca^{2+}, Fe^{3+}, Al^{3+}, and Cu^{2+} and retained 86.2% to 98.7% residual activities.[1]

Activities of BG1 and BG2 were increased by 18% and 30.6% in the presence of Fe^{2+} but inhibited by Fe^{3+} with 87.8% and 45.5% residual activities, respectively. This suggests that different iron valences (Fe^{2+} and Fe^{3+}) show different influences on the activities of the β-glucosidases.[19] Inactivation of β-glucosidase by Fe^{2+} was observed in many strains: 20% residual activity toward pNPG in *Talaromyces thermophilus*, 40% to 70% in yeast *Pichia etchellsii*, about 90% in *Periconia sp.*, and even complete inhibition in *Penicillium pinophilum*,[1,25−27] which differed from the properties of β-glucosidases from *T. asahii*. In addition, β-glucosidase activity of filamentous fungus (*Stachybotrys microspora*) was upgraded by Fe^{2+} to 161%, while it was strongly inhibited by Fe^{3+}.[28] This is in accordance with the effect of Fe^{2+} on β-glucosidases from *T. asahii*.

Table 2 also shows the effects of different inhibitors on the activities of two β-glucosidases. Alcohols showed almost no influence on enzyme activities. These results suggest that β-glucosidases from *T. asahii* have better resistance to alcohol than β-glucosidase from *A. niger*.[29] SDS stimulated the activity of BG1 by 8.32%, whereas it strongly inhibited the activity of BG2 up to approximately 80%. This result suggests that BG1 has resistance to some anionic detergents, such as SDS. SDS resistance was a striking property of purified BG1 in comparison with that of some other β-glucosidases, which maybe make it a potential candidate for industrial applications even under certain chemical circumstances.[1,5,9,30]

In the test for the presence of an essential disulfide bond, β-mercaptoethanol (1% v/v) exhibited a different effect on the two β-glucosidases. Only the activity of BG1 was increased 16% by β-mercaptoethanol, which suggests the probable absence of a disulfide bond in the active catalytic site. The purified BG1 and BG2 were also less susceptible to the nonionic detergent Triton X-100. The chelating agent, ethylenediaminetetraacetic acid (EDTA), inhibited the enzymes severely. The susceptibility of the two β-glucosidases to EDTA is in accordance with Matern's result but in contrast to Saha's findings.[31,32]

Substrate Specificity of β-Glucosidase. The substrate specificity toward oligosaccharides and aryl-β-glycosides of β-glucosidases from *T. asahii* were investigated (Table 3). Among the aryl-β-glycoside substrates, pNPG was the preferred substrate for BG1 and BG2. Moreover, BG2 also exhibited higher affinity to pNPF (47.25%) than BG1 (1.93%). BG1 and BG2 both displayed the highest hydrolysis activity toward gentiobiose (BG1 496.62%, BG2 487.52%), followed by maltopentaose (BG1 312.19%, BG2 313.39%) and maltotriose (BG1 228.11%, BG2 242.97%). The enzymes exhibited high affinity to gentiobiose and very low affinity to trehalose among disaccharide substrates, which was consistent with Gao's research.[33]

As shown in Table 3, among the β-1,6 linkage (gentiobiose), β-1,2 linkage (sophorose), β-1,3 linkage (laminaribiose), β-1,4 linkage (cellobiose), and α-1,1 linkage (trehalose), the two β-glucosidases were highly active on the linkage of glucose-β-(1 → 6)-glucose among these substrates. The higher preference to the β-1,6 linkage was in accordance with the report of Zhao.[34]

The β-glucosidases from *T. asahii* also exhibited more preference to the maltose series between two groups of chain-length substrates (maltose to maltopentaose, cellobiose to cellotetraose) (Table 3). BG1 and BG2 both showed similar affinity trends to different chain-length substrates. This preference can be related to the structural features of these substrates and the properties of the β-glucosidases.[35]

Table 3. Relative Activity of β-Glucosidases on Various Substrates

substrate		type of bond	activity (%)[a] BG1	BG2
4-nitrophenol glycosides[b]	4-nitrophenyl-β-D-glucopyranoside	β-1,4	100	100
	4-nitrophenyl-β-D-fucopyranoside	β-1,4	1.93 ± 0.69	47.25 ± 0.87
	4-nitrophenyl-β-D-galactopyranoside	β-1,4	1.53 ± 0.98	2.08 ± 0.76
saccharides[c]	sophorose	β-1,2	46.24 ± 0.95	37.13 ± 2.13
	laminaribiose	β-1,3	6.34 ± 0.01	12.19 ± 0.15
	gentiobiose	β-1,6	496.62 ± 9.6	487.52 ± 8.76
	trehalose	α-1,1	2.90 ± 0.12	2.82 ± 0.08
	maltose	α-1,4	161.44 ± 9.23	176.07 ± 8.31
	maltotriose	α-1,4	228.11 ± 9.45	242.97 ± 10.51
	maltopentaose	α-1,4	312.19 ± 12.70	313.39 ± 15.59
	cellobiose	β-1,4	97.34 ± 3.10	93.67 ± 0.81
	cellotriose	β-1,4	117.15 ± 0.83	114.69 ± 0.81
	cellotetraose	β-1,4	110.68 ± 1.94	84.72 ± 0.96
	cellulose	β-1,4	29.25 ± 2.18	13.70 ± 1.30

[a] Values were expressed as the means ± SD, $n = 3$. [b] The hydrolysis of the substrates in a 0.1 M citrate−phosphate buffer (pH 5.0) at 50 °C was measured and the activity expressed relative to the activity measured on pNPG (100%). [c] The hydrolysis of the substrates in a 0.1 M citrate−phosphate buffer (pH 5.0) at 50 °C was measured and the activity expressed relative to the activity measured on gentiobiose (100%).

Table 4. Contents of Estimated Secondary Structures of β-Glucosidases of *T. asahii*

	α-helix (%)	β-sheet (%)	turns	random coil (%)
BG1	2.81	48.9	23.1	25.2
BG2	0	10.9	28.3	60.8

Besides exhibiting different hydrolysis activities to soluble substrates, both enzymes also had little hydrolysis ability to partly solubilize cellulose. Broad substrate specificity is a characteristic shared by several fungal β-glucosidases.[2,14,19,22]

Circular Dichroism Studies. The secondary structures of the β-glucosidases were investigated by circular dichroism measurements. As shown in Table 4 and Figure 6, there were many differences between BG1 and BG2 in their secondary structures. It is commonly known that the negative peak between 200 and 205 nm in circular dichroism analysis is a characteristic of random coil conformation.[36] In the present study, BG2 exhibited predominantly random coil conformation (60.8%), as evidenced by the negative peak in the 200−205 nm wavelength regions in far-UV CD spectra. A positive peak between 190 and 220 nm and single negative peak between 210−215 nm are the characteristic peaks of a β-sheet and typical for a very highly β-sheet rich protein.[37] In addition, the peak at 218 nm indicates a typical antiparallel β-sheet profile.[38] All these indicated a predominance of β-sheet structure in BG1 (48.9%). The SDS-resistant and high β-sheet secondary structure of BG1 suggests higher kinetic stability than BG2.[30]

Transglycosylation Properties. BG1 and BG2 were both subjected to reverse synthesis and transglycosylation assays, but only BG1 clearly exhibited these abilities. A single peak (13.99 min) of glucose free of any di-, tri-, or oligosaccharide mixture at time 0 h incubation period was identified (Figure 7a−d) by analysis of the products from the former reaction. After 4 h, peaks of cello-oligosaccharides at 8.58 min, 9.42 min, and 10.96 min were visible, and after 8 h, three peaks at 8.03 min, 8.84 min, and 10.32 min corresponding to cellotetraose, cellotriose, and cellobiose, respectively, were observed. The maximum yield of

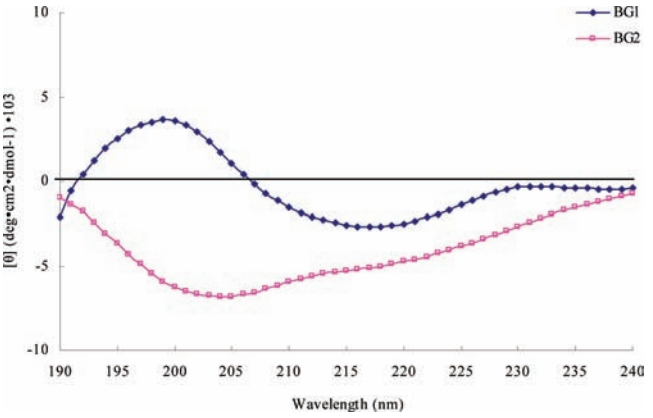

Figure 6. Circular dichroic spectra of β-glucosidases from *T. asahii*. Protein CD spectra were analyzed using Dichroweb to produce the indicated protein secondary structure contents. Experimental conditions: 10 mM phosphate buffer, pH 5.0, and 20 °C. Enzyme concentration was 30 μg/mL.

cello-oligosaccharides was estimated to be about 6.52% calculated by the peak area from the HPLC chromatogram in 8 h under experimental conditions. Incubating glucose without BG1 under the same conditions (control experiments) did not result in any detectable oligosaccharides or loss of glucose. In order to investigate the transglycosylation alone of BG1, the transglycosilation assay was performed with cellobiose as the substrate (Figure 7e−h). After 6 h of incubation, a peak of cellotetraose at 7.85 min was visible accompanied with the appearance of glucose (12.51 min) in comparison with Figure 7e. After 12 h, three peaks at 7.82 min, 8.58 min, and 12.47 min corresponding to cellotetraose, cellotriose, and glucose, respectively, were observed. The maximum yield of cellotetraose and cellotriose was estimated to be 1.54% and 0.86%, respectively. No oligosaccharides, apart from cellobiose or glucose, were detected in the controls. The appearance of free glucose was derived from the hydrolysis of

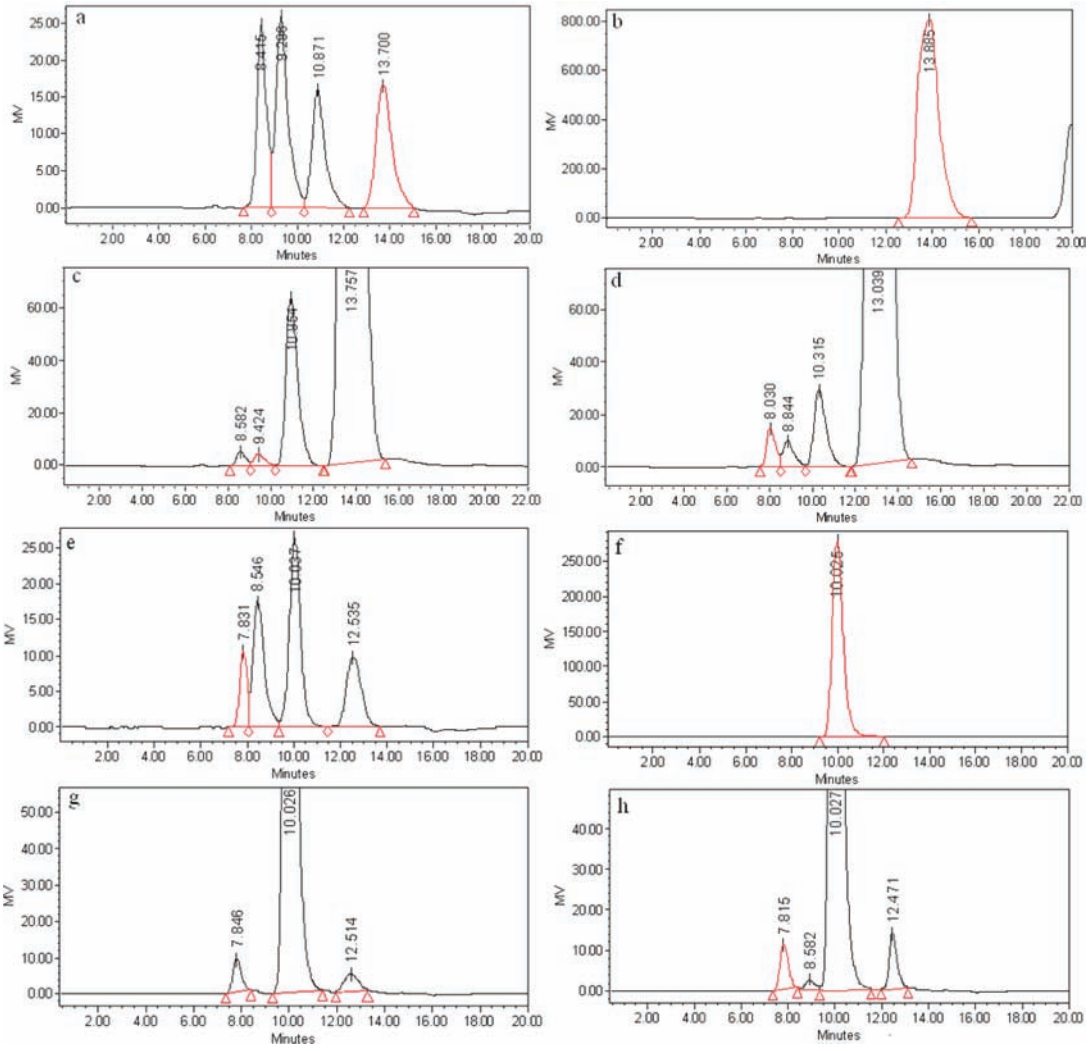

Figure 7. Reverse hydrolysis and transglycosylation assay. Reverse hydrolysis was carried out by incubating the enzyme with glucose for 0 h (b), 4 h (c), and 8 h (d). Standard samples of glucose (13.76 min), cellobiose (10.87 min), cellotriose (9.29 min), and cellotetraose (8.42 min) were run as controls (a) for reverse hydrolysis. A transglycosylation reaction was carried out by incubating the enzyme with cellobiose for 0 h (f), 6 h (g), and 12 h (h). Standard samples of glucose (12.54 min), cellobiose (10.04 min), cellotriose (8.55 min), and cellotetraose (7.83 min) were run as controls (e) for the transglycosylation reaction.

cellobiose. This phenomenon indicated that the glucose was simultaneously involved in two reactions, hydrolysis and transglycosylation. The obtainment of cello-oligosaccharides suggested that BG1 actually had the ability of transglycosylation.

Enzyme-catalyzed synthesis of oligosaccharides is a useful method for the selective formation of well-defined oligosaccharides.[39] Because β-glucosidase is widely available, is less expensive, and does not require expensive sugar nucleotide donors than glycosyltransferases, it is especially attractive for oligosaccharide synthesis.[11,35] Because of its unique characteristics, such as the higher resistance to most of the metal ions and chemical reagents used in this study and the transglycosylation activity, BG1 from *T.asahii* appears to represent a novel β-glucosidase. These properties mark BG1 as a potential enzyme for a wide range of industrial applications.

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