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Screening and Characterization of an Enzyme with β-Glucosidase Activity from Environmental DNA

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Abstract A novel β-glucosidase gene, *bglA*, was isolated from uncultured soil bacteria and characterized. Using genomic libraries constructed from soil DNA, a gene encoding a protein that hydrolyzes a fluorogenic analog of cellulose, 4-methylumbelliferyl β-D-cellobioside (MUC), was isolated using a microtiter plate assay. The gene, *bglA*, was sequenced using a shotgun approach, and expressed in *E. coli*. The deduced 55-kDa amino acid sequence for *bglA* showed a 56% identity with the family 1 glycosyl hydrolase *Chloroflexus aurantiacus*. BglA included two conserved family 1 glycosyl hydrolase regions. When using *p*-nitrophenyl-β-D-glucoside (*p*NPG) as the substrate, the maximum activity of the purified β-glucosidase exhibited at pH 6.5 and 55°C, and was enhanced in the presence of Mn^{2+}. The K_m and V_{max} values for the purified enzyme with *p*NPG were 0.16 mM and 19.10 μmol/min, respectively. The purified BglA enzyme hydrolyzed both *p*NPG and *p*-nitrophenyl-β-D-fucoside. The enzyme also exhibited substantial glycosyl hydrolase activities with natural glycosyl substrates, such as sophorose, cellobiose, cellotriose, cellotetraose, and cellopentaose, yet low hydrolytic activities with gentiobiose, salicin, and arbutin. Moreover, BglA was able to convert the major ginsenoside Rb_1 into the pharmaceutically active minor ginsenoside Rd within 24 h.

Keywords: β-Glucosidase, environmental DNA, ginsenoside

Many useful soil microorganisms have already been isolated as pure cultures, and applied to various industrial purposes. Nonetheless, the full biotechnological potential of the highly diverse and versatile range of microbial enzymes has not yet been fully explored [1, 8, 9, 26, 27, 36, 37]. Thus, to understand the ecological and biotechnological potential of many uncultured microorganisms, metagenomics has recently been applied to environmental samples as a cultivation-independent approach [10–13, 19, 21, 33, 39]. With this method, DNA is directly extracted from environmental samples and cloned into appropriate vectors to construct genomic libraries. These libraries can then be screened and analyzed for novel functional enzymes [16, 43].

β-Glucosidases (EC 3.2.1.21) catalyze the cleavage of the glycosidic bonds of disaccharides, oligosaccharides, and alkyl- and aryl β-glucosides [20, 41], and have been classified in family 1 and family 3 of the glycosyl hydrolase families based on their amino acid sequence similarities [17]. Family 1 enzymes mostly include bacterial, archaeal, plant, and animal β-glucosidases, whereas family 3 includes some bacterial and all yeast and fungi glucosidases [5, 14, 18, 28, 29, 34, 38].

β-Glucosidases have many potential applications in various biotechnological processes. For example, these enzymes are involved in the hydrolysis of bitter compounds during fruit-juice extraction and the release of aromatic compounds from flavorless precursor glucosides [31], along with cellulose hydrolysis and ethanol production [3, 25, 42].

However, one important β-glucosidase application is the conversion of more common ginsenosides (Rb_1, Rb_2, and Rc) into rare ginsenosides (Rd, Rg_3, Rh_2, and compound K) [6, 23, 45, 46]. Ginseng roots contain various pharmaceutical component ginsenosides, and wild and red ginseng include small percentages of rare ginsenosides (Rd, Rg_3, Rh_2, and compound K), which have recently attracted much interest owing to their drug-related activities. For example, ginsenoside Rd prevents kidney injury by chemical drugs and protects the neural systems against neurotoxicity [24]. Ginsenosides Rb_1, Rb_2, and Rc are absorbed by intestinal microflora in the human body as compound K *via* ginsenoside Rd [4], where this biological transformation of ginsenosides has a significant impact on absorption, regardless of personal intestinal microflora. The rare ginsenosides can

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be produced by the biological hydrolysis of the sugar moieties from the more common ginsenosides (Rb_1, Rb_2, and Rc). Ginsenoside Rb_1 is the most abundant of all the ginsenosides, and its structure can be easily converted into ginsenoside Rd by the hydrolysis of one glucose moiety [23].

Accordingly, this study constructed metagenome libraries from environmental soil samples to screen for novel β-glucosidase-encoding genes using a microtiter plate assay. As a result, the *bglA* gene was isolated and characterized as regards its β-glucosidase activities and a number of other properties. BglA was found to exhibit different properties compared with other known β-glucosidases and a novel substrate hydrolysis profile.

MATERIALS AND METHODS

Construction of Metagenome Library

Soils were collected from the "Upo" wetland in Korea, and 5 g of samples added to 15 g of glass and ceramic beads (Mobio, U.S.A.) in an extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, and 1% hexadecyltrimethylammonium bromide) containing 100 μl proteinase K (20 mg/ml). The samples were then vortexed at room temperature for 10 min at 1,400 rpm, 1.5 ml of 20% SDS added, and the samples incubated at 65°C for 2 h. Thereafter, the supernatants were collected and precipitated as described previously [44].

Because of humic compounds in the samples, the DNA preparations were further purified by agarose gel electrophoresis, which also facilitated size fractionation of the DNA. DNA fragments within a size range of 30–50 kb were then used for an end-repair reaction based on an EndRepair kit, and ligated into a pEpiFOS-5 fosmid vector (Epicentre, U.S.A.) according to the manufacturer's recommendations. *In vitro* packaging was performed with a MaxPlax lambda packaging extract kit (Epicentre, U.S.A.). Finally, the products were transformed into *E. coli* EPI100.

Library Screening and Sequence Analysis

The metagenome libraries were assayed using a fluorogenic substrate analog of cellulose. The libraries were also replicated into 96-well plates for a 4-methylumbelliferyl β-D-cellobioside (MUC) hydrolysis assay. After adding the substrate (0.1 mM MUC), the plates were incubated at 28°C and examined daily with UV light. Pure strains of positive clones were obtained from the fluorescing wells. DNA fragments of these positive clones were then generated by sonication (Misonix Sonicator 3000, 10 s, 0.5 output), end-repaired, cloned into a pUC118/HincII vector, and screened using the same MUC hydrolysis assay. DNA sequence reactions were carried out in a PTC-200 Thermo cycler (MJ Research, U.S.A.) with an ABI PRISM Bigdye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, U.S.A.) according to the manufacturer's instructions.

Enzyme Overexpression and Purification

The putative β-glucosidase gene was amplified from one of the MUC-positive clones (pCE92) using the primers 5'-TCGGATCCATGCCAGCCGGCCTGCTGATC-3' with a BamHI site at the 5'-end and 5'-GCAAGCTTAATAGCG-GGCGCGGCTAGCCC-3' with a HindIII site at the 3'-end. The amplified DNA was then ligated into BamHI and HindIII double-digested pET32a(+) (Novagen), and the construct (pEGLU92) transformed into *E. coli* BL21(DE3) cells. To trap the His-tagged protein, the crude extract was purified using an Ni-NTA agarose slurry according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Enzyme Activity Determination

The enzyme assays were carried out with *p*-nitrophenyl-β-D-glucoside (*p*NPG) as the substrate at 55°C, as described by Marques *et al.* [28]. Briefly, an appropriate amount of the enzyme, 1 mM *p*NPG, and a 50 mM sodium phosphate buffer (pH 6.0) were mixed in a final volume of 150 μl. After the incubation period, the reaction was stopped with 100 μl of 1 M Na_2CO_3 and the absorbance measured at 420 nm. One unit of β-glucosidase was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenyl per min under the assay conditions.

The substrate specificity was tested by replacing *p*NP-glucoside with other *p*-nitrophenol-coupled compounds. The activity on natural glycosides was analyzed by measuring the released glucose with a glucose assay kit (Sigma, U.S.A.).

The effects of pH and temperature on the β-glucosidase activity were examined using the purified recombinant enzyme. To determine the optimal pH, the following buffers were used for different pH ranges: pH 4.5 to 6.0, 50 mM sodium acetate; pH 5.5 to 8.0, 50 mM sodium phosphate; pH 7.5 to 9.0, 50 mM Tris-HCl. To determine the optimal temperature, the enzyme mixtures were incubated at temperatures from 35°C to 80°C for 15 min. Thermostability data were obtained by preincubating the enzyme at various temperatures, as above, and then measuring the residual activities under the same standard assay conditions. The effects of various metal ions were measured at 5 mM concentrations.

PAGE and Zymogram Analysis

The purified BglA was separated on an 8% SDS-PAGE gel followed by Coomassie Blue R-250 staining. For a zymogram analysis, the native 8% gel was run at 4°C with the purified protein. The gel was then incubated in 1 mM

4-methylumbelliferyl β-D-glucoside (MUG) at 50°C for 10 min, and the enzyme activity in the gel visualized under UV by observing the fluorescence generated by the hydrolytic product 4-methylumbelliferone.

Thin-Layer Chromatography (TLC) Analysis and HPLC Analysis

One unit of β-glucosidase and 1 mM of ginsenoside Rb_1 were dissolved in 1.5 ml of a 0.1 M sodium phosphate buffer (pH 6.0), and the reaction mixture incubated at 50°C for 24 h. The prepared samples were then spotted on a silica gel 60 F_{254} plate, and the plate developed with a solvent mixture of $CHCl_3$-MeOH-H_2O (65:35:10, v/v/v, lower phase). Thereafter, the plate was soaked rapidly into 5% H_2SO_4 and heated at 110°C for 5 min. The same reaction mixture was separated by HPLC using an Alltech Prevail Carbohydrate ES column under gradient conditions with acetonitrile-water-isopropanol (80:5:15, v/v/v) (solvent A) and acetonitrile-water-isopropanol (80:20:15, v/v/v) (solvent B).

Database Submission

The nucleotide sequence for clone pUGLU92 was deposited in the GenBank database under the accession no. DQ842022.

RESULTS

Screening of Environmental DNA Libraries

Metagenome libraries were constructed for soil samples, taken from the Upo wetlands in Korea, based on direct DNA extraction and purification. To test the quality of the libraries, 30 clones were randomly selected. The average insert size was ~35 kb and the percentage of plasmids containing inserts was approximately 93%. The fosmid libraries of metagenomic DNA were screened for clones expressing β-glucosidase activity on LB media containing 0.1 mM MUC. From the initial screening, 5 putative β-glucosidase-active clones were identified among 14,000 metagenome clones. These 5 fosmid clones expressing β-glucosidase activity were then subcloned using the shotgun approach, and the subclones screened again using the same MUC assay. Finally, one positive subclone (pUGLU92) containing a 9,252 bp DNA fragment was sequenced and analyzed.

Molecular Analysis of β-Glucosidase

The insert DNA sequence of pUGLU92 was compared with the sequences in the GenBank database using a BLAST algorithm [2], and the search revealed a significant homology

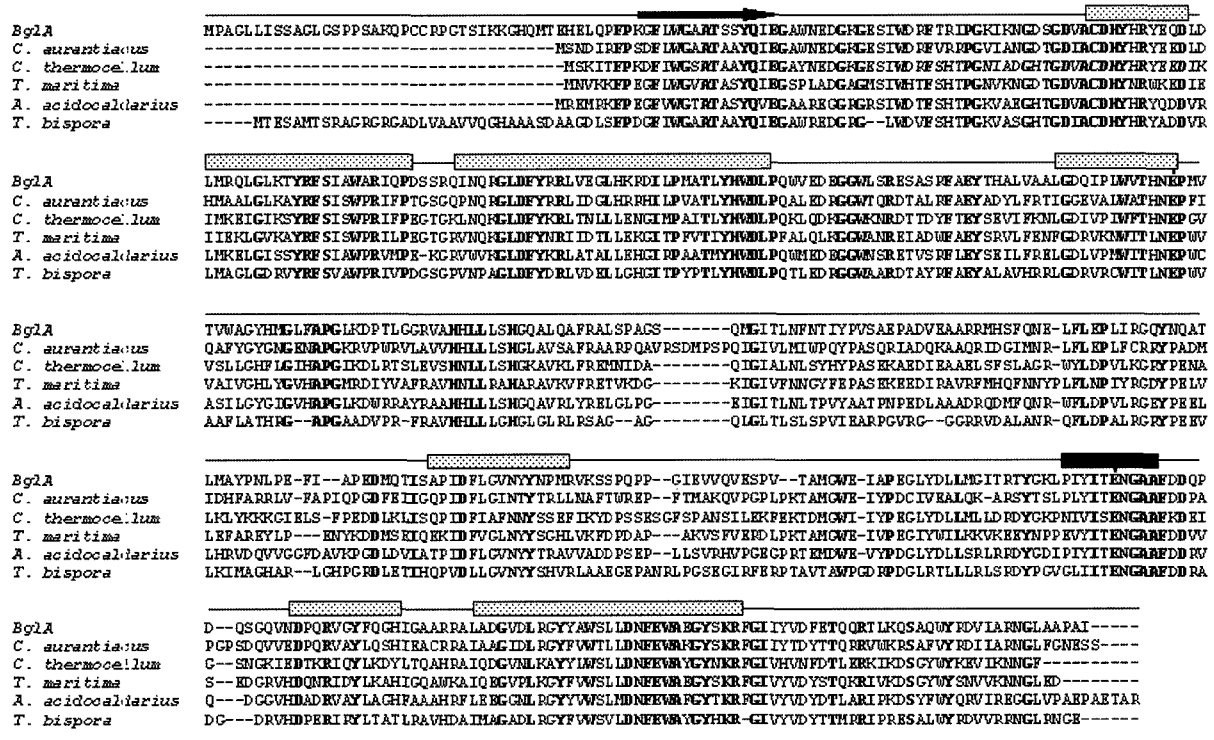

Fig. 1. Multiple alignment of *bglA* amino acid sequences.

The ClustalW algorithm was applied to the amino acid sequences of β-glucosidase from C. aurantiacus (GenBank Accession No. ZP00766738), C. thermocellum (P26208), T. maritima (Q08638), A. acidocaldarius (AAZ81839), and T. bispora (P38645) using the BLOSUM62 matrix. Identical residues are indicated by bold-type. A glycoside hydrolase N-terminal domain (arrow) and glycoside hydrolase active motifs (shaded bars) are shown on top of the deduced bglA amino acid sequences. The glycoside hydrolase active sites are represented by a filled box. Two glutamate residues with acid/base- and nucleophile-motifs are represented by shaded characters.

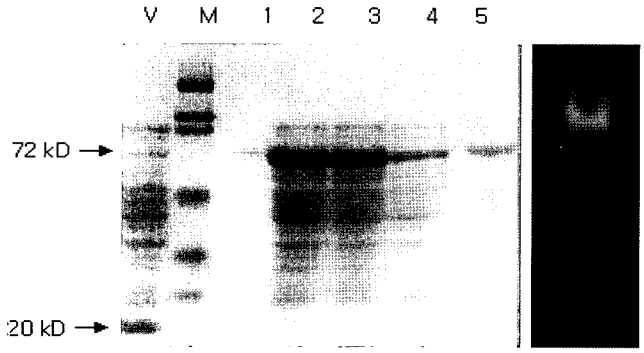

Fig. 2. SDS-PAGE analysis of purified BglA and native-PAGE analysis for detection of BglA activity.
β-Glucosidase activity was detected using MUG as the substrate. M, broad range marker; 1, culture supernatant; 2, cell lysate in 20 mM imidazole solution; 3, wash-out fraction; 4, fraction eluates with 50 mM imidazole; 5, fraction eluates with 250 mM imidazole.

between one ORF (1,455 bp) in pUGLU92 and a family 1 glycosyl hydrolase from *Chloroflexus aurantiacus* (56% identity, 67% positives). Therefore, the annotation *bglA* was given to the ORF sequence. *bglA* contained two conserved regions in common with family 1 glycosyl hydrolases [15], including typical acid/base- and nucleophile-residues found in glycosyl hydrolases [32, 40] (Fig. 1). The molecular mass of the translated protein was estimated to be 55,506 Da.

Expression and Purification of BglA

The *bglA* gene was heterologously expressed in the pET32a(+) expression vector, and then the recombinant plasmid with the *bglA* ORF inside, pEGLU92, was introduced into *E. coli* BL21(DE3). The expressed protein was purified to homogeneity from the cellular extracts of this strain using an Ni-NTA agarose slurry. The molecular mass of the expressed BglA and His-tag was 72 kDa, which corresponded well with the predicted molecular mass for BglA from the DNA sequences (55 kDa). To characterize the β-glucosidase activities, a direct activity staining technique was used based on MUG-PAGE [41]. A fluorescent band appeared around the expressed protein where the MUG was degraded (Fig. 2), indicating the glycosyl hydrolase activity of the BglA.

Effect of pH, Temperature, and Metal Ions on Activity of BglA

The effect of pH on the BglA activity against *p*NPG was determined at 55°C in various buffers ranging from pH 4.0 to 9.0. The maximal *p*NPG degradation activity was observed

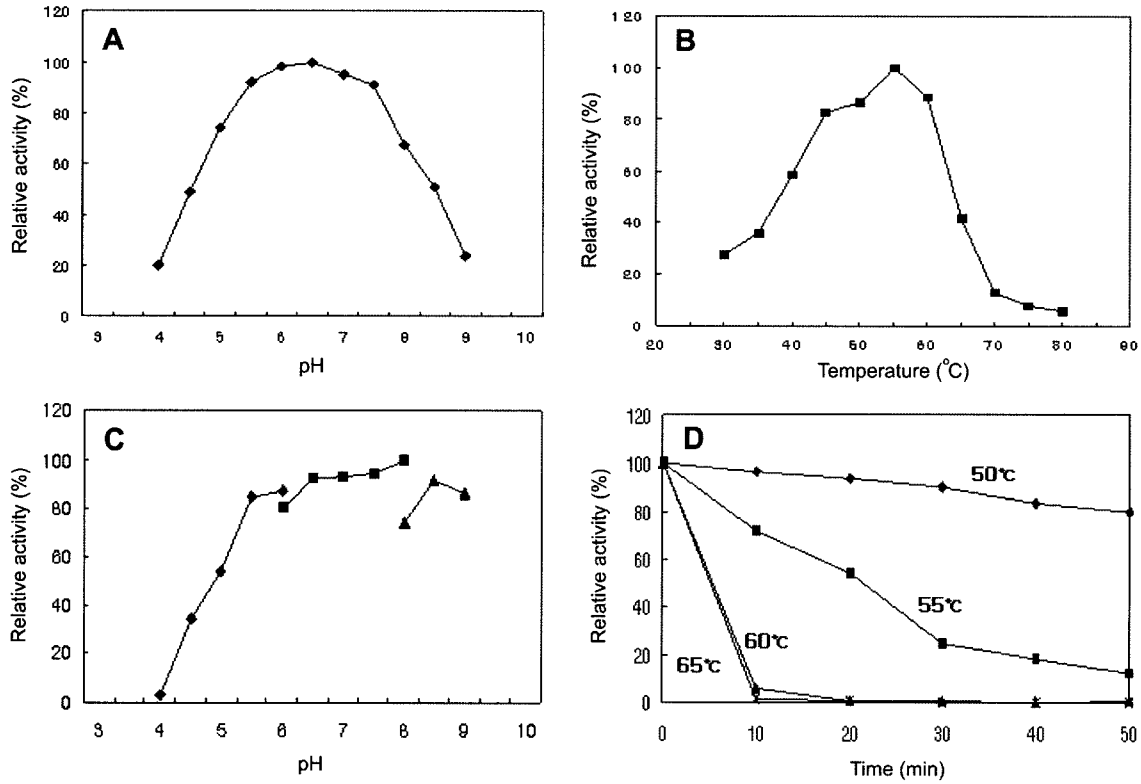

Fig. 3. Effect of pH and temperature on enzyme activity.
A. Optimum pH for BglA activity, where enzymatic activity was assayed at 55°C for 15 min in indicated sodium phosphate buffers; **B.** Optimum temperature for BglA activity, where enzyme activity was assayed at pH 6.5 for 15 min at indicated temperatures; **C.** pH stability of enzyme; **D.** Thermostability of enzyme.

at pH 6.5 (Fig. 3), and more than 50% of the enzymatic activity was retained within a pH range of 4.5 to 8.5. Similarly, the temperature dependence of the BglA activity toward *p*NPG was determined by measuring the degradation of *p*NPG at various temperatures at pH 6.5, and the maximal activity was observed at 55°C. Since the industrial application of β-glucosidase requires a thermostable enzyme, and temperature stability of BglA was also measured. The thermostability data were obtained by preincubating BglA at various temperatures, including the optimal temperature 55°C, and then measuring the residual *p*NPG hydrolyzing activity using the same standard *p*NPG assay. At 50°C, most activity (82%) was retained even after 60 min of incubation, whereas at 55°C, about 80% of the β-glucosidase activities were lost during the same time period. The stability test at different pH levels showed that the purified enzyme was broadly stable within a pH range of 5.5 to 9.0. When measuring the β-glucosidase activity at pH 6.5 and 55°C in the presence of various metal ions (data not shown), divalent cations, such as Mg^{2+}, Mn^{2+}, and Cu^{2+}, had positive effects, whereas Zn^{2+}, Ca^{2+}, Co^{2+}, and Fe^{2+} inhibited the enzyme activity.

Table 1. Substrate specificity of BglA.

Substrates	Relative activity (%)
Natural glycosides	
Saccarides (1 mM)	
Cellobiose [β(1,4)Glc]	49
Cellotriose [β(1,4)Glc]	59
Cellotetraose [β(1,4)Glc]	81
Cellopentaose [β(1,4)Glc]	84
Gentiobiose [β(1,6)Glc]	9
Sophorose [β(1,2)Glc]	102
Sucrose	11
Lactose	9
Maltose	10
Melibiose	11
Aryl-glycosides	
Salicin	2
Arbutin	10
Synthetic Aryl-glycosides (1 mM)	
p-NP-β-D-glucoside	100
p-NP-β-D-fucoside	220
p-NP-α-D-glucoside	0
p-NP-α-L-fucoside	0
p-NP-β-D-cellobioside	26.2
p-NP-α-D-cellobioside	5.3
p-NP-β-D-galactoside	30
p-NP-α-D-galactoside	0
p-NP-β-D-lactoside	13.3
p-NP-β-D-mannoside	3
p-NP-α-D-mannoside	0
p-NP-β-D-xyloside	2
p-NP-β-D-glucuronide	0
p-NP-α-L-arabinoside	0
p-NP-α-L-rhamnoside	0
p-NP-N-acetyl-β-D-glucosamine	0
p-NP-N-acetyl-β-D-galactosamine	0

The purified BglA was assayed for its activity using different saccharides and aryl-glycosides (1 mM) in a Na-phosphate buffer (50 mM, pH 6.5).

Substrate Specificity of BglA

The action of the purified BglA was tested over a large number of substrates with α and β configurations (Table 1). Whereas the enzyme efficiently hydrolyzed *p*NPG and *p*NPF, a lower level hydrolysis was observed on *p*NP-β-cellobioside (26%), *p*NP-β-galactoside (30%), *p*NP-β-lactoside (13%), *p*NP-β-xyloside (2%), and *p*NP-β-mannoside (3%). Interestingly, *p*NP-β-glucuronide, *p*NP-N-acetyl-β-glucosamine, and *p*NP-N-acetyl-β-galactosamine did not show any activity, even though they are all β configurations. In addition, hydrolytic activity was observed on sophorose, cellobiose, cellotriose, cellotetraose, and cellopentaose, whereas low hydrolytic activity was observed on gentiobiose, salicin, and arbutin (Table 1). Therefore, these results indicated that BglA hydrolyzes both β-(1→4)-glucosides and β-(1→2)-glucosides. The kinetic parameters, K_m and V_{max}, for the purified β-glucosidase were obtained on *p*NPG using a substrate concentration range of 0.1–2 mM. The K_m values were then determined by analyzing the slopes of Lineweaver-Burk plots, which revealed a linear substrate response over the concentration range. The K_m and V_{max} values of the BglA enzyme for *p*NPG were 0.16 mM and 19.10 μmol/min, respectively. However, the K_m and V_{max} values for *p*NPF were 0.67 mM and 59.66 μmol/

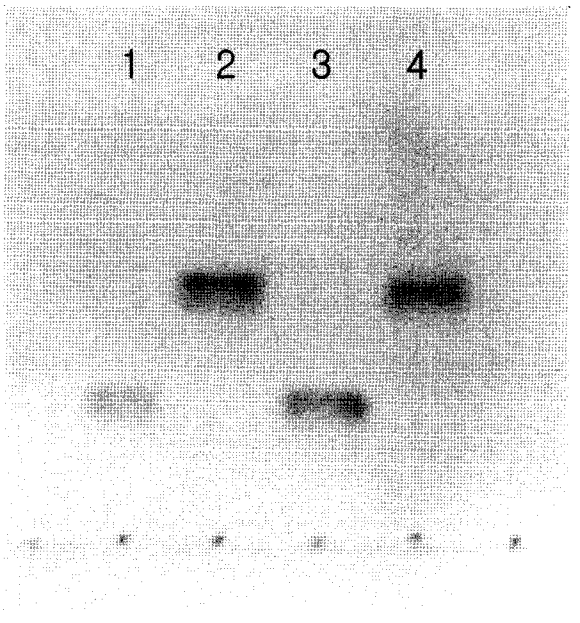

Fig. 4. Conversion of Rb_1 into Rd using β-glucosidase.
1, Rb_1; 2, Rd; 3, initial reaction mixture; 4, reaction mixture after 24 h.

min, respectively, indicating a weak binding with *p*NPF. Thus, when considering all the results, it would appear that BglA is a novel β-glucosidase isolated from a soil metagenome.

Conversion of Ginsenoside Rb1 by BglA

The purified BglA was further assayed to verify its hydrolytic activity for converting ginsenoside Rb_1. After incubating BglA with ginsenoside Rb_1 at 50°C for 24 h, a significant conversion of ginsenoside Rb_1 into Rd was observed. (Fig. 4). An HPLC analysis further verified the almost complete conversion of ginsenoside Rb_1 into Rd (Fig. 5). One of the new peaks had a retention time consistent with that of ginsenoside Rd (the main product). Therefore, these results imply that the complete disappearance of ginsenoside Rb_1 was accompanied by the accumulation of ginsenoside Rd in the indicated reaction mixture.

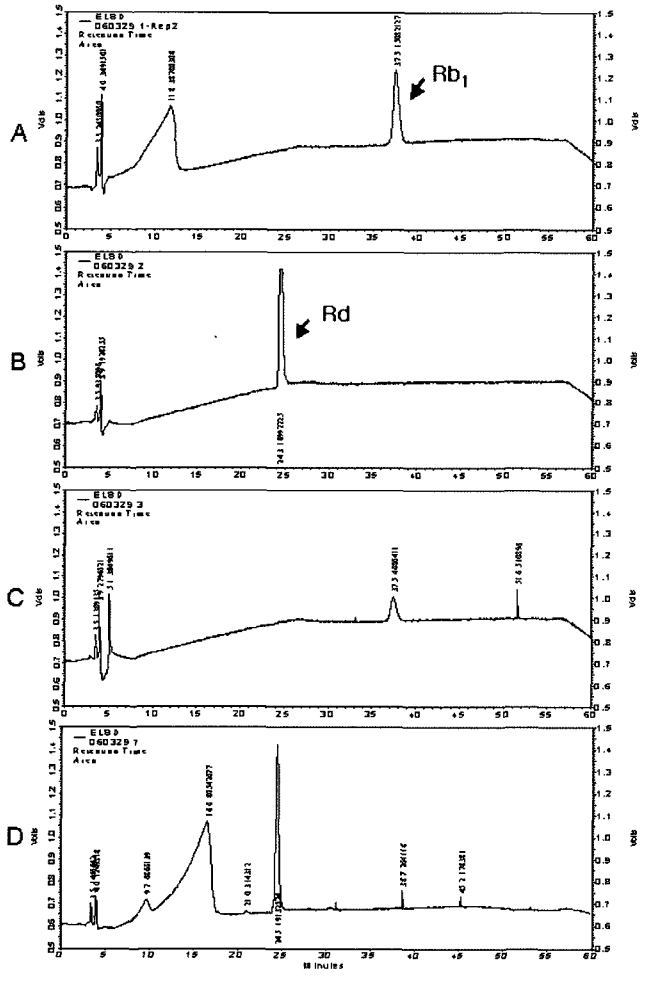

Fig. 5. HPLC analysis of Rb_1 into Rd using BglA.
A. Rb_1 control compound; **B.** Rd control compound; **C.** Initial reaction mixture; **D.** Reaction mixture after 24 h. The ratios of solvent A/solvent B were 70/30, 0/100, 0/100, and 70/30 at running times of 0, 20, 50, and 60 min, respectively, at a flow rate of 0.8 ml/min with ELSD detection.

DISCUSSION

β-Glucosidases are divided into three groups on the basis of their substrate specificity. The first group is known as aryl-β-glucosidases owing to their strong affinity for aryl-β-glucosidase. The second group consists of cellobiases that only hydrolyze oligosaccharides. The third group is broad-specific β-glucosidases that exhibit activity on a wide range of substrates, and are the most commonly observed biological form of β-glucosidases [32]. Thus, in an attempt to isolate novel β-glucosidases from uncultured soil microorganisms, the present study constructed a genomic library from an environmentally well-preserved wetland in Korea. As a liquid microtiter assay is known to be more sensitive for detecting low-level MUC hydrolysis than a clearing zone assay on agar plates [7], a 96-well liquid β-glucosidase assay was selected for the screening method. Among 10,000 metagenome clones, five positive clones were isolated. Although all 5 clones showed significant MUC hydrolysis activities, only 1 insert was found to contain an open reading frame with a homology to known β-glucosidases. As yet, it is still unclear why the other 4 clones showed hydrolase activity without any known β-glucosidase genes. Based on its amino acid sequence, *bglA* was identified as a family 1 glycosyl hydrolase [32]. Therefore, this would appear to be the first report of the isolation of a β-glucosidase identified as a family 1 glycosyl hydrolase from a metagenome library.

The purified BglA hydrolyzed β-(1→4)-, β-(1→2)-glycosides, as well as synthetic aryl-β-glycosides. The enzyme also exhibited no activity on *p*NP-α-D-glycosides in contrast to *p*NP-β-D-glycosides. Moreover, BglA exhibited low activity on β-(1→6)-glycosides in contrast to other β-glucosidases [30]. Although BglA was active with synthetic aryl-β-glucosides, it showed a low activity against natural aryl-glucosides, such as salicin and arbutin. BglA was able to hydrolyze cellobiose, cellotriose, cellotetraose, and cellopentaose, and its activity was generally enhanced as the chain length increased. Moreover, a two-fold higher activity was shown towards pNPF when compared with other substrates, implying that BglA includes β-fucosidase activity in addition to β-glucosidase. β-Glucosidases sharing β-fucosidase activities have been previously observed [22, 35]. Yet, in contrast to the published characteristics for the strict β-fucosidase from *Lactuca sativa* latex, which includes activities towards both *p*NPF and *p*NP-α-L-fucoside, the purified BglA exhibited no activity towards *p*NP-β-fucoside, indicating that BglA has different properties from other β-glucosidases and a novel substrate hydrolysis profile.

Currently, β-glucosidases are actively applied to the production of value-added products in various areas, including pharmaceuticals. Thus, to evaluate the practical application of the novel BglA, a test conversion of ginsenosides was performed. The microbial conversion of ginsenoside Rb_1

using aerobic bacteria isolated from a ginseng field has been previously reported [23]. However, Rd conversion has not yet been characterized at an enzymatic level. Until now, only one enzyme has been shown to convert ginsenoside Rb_1 into ginsenoside Rd, and this enzyme was purified from human intestinal bacteria [4]. Therefore, the present conversion of Rb_1 into Rd by BglA demonstrated that the enzyme appeared to include significant β-glucosidase activity, allowing Rb_1 to be almost completely converted into Rd. Therefore, this study is the first use of metagenome libraries to isolate an enzyme capable of converting ginsenoside Rb_1.

In conclusion, a novel β-glucosidase-encoding gene, *bglA*, was isolated from a soil metagenome library. However, further studies of its three-dimensional structure, kinetics, and *in vitro* evolution are required to explain the uncommon properties of BglA.

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