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Characterization of a Novel β-Glucosidase-Like Activity from a Soil Metagenome

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We report the cloning of a novel β-glucosidase-like gene by function-based screening of a metagenomic library from uncultured soil microorganisms. The gene was named *bgl1C* and has an open reading frame of 1,443 base pairs. It encodes a 481 amino acid polypeptide with a predicted molecular mass of about 57.8 kDa. The deduced amino acid sequence did not show any homology with known β-glucosidases. The putative β-glucosidase gene was subcloned into the pETBlue-2 vector and overexpressed in *E. coli* Tuner (DE3) pLacI; the recombinant protein was purified to homogeneity. Functional characterization with a high performance liquid chromatography method demonstrated that the recombinant Bgl1C protein hydrolyzed D-glucosyl-β-(1-4)-D-glucose to glucose. The maximum activity for Bgl1C protein occurred at pH 8.0 and 42°C using *p*-nitrophenyl-β-D-glucoside as the substrate. A $CaCl_2$ concentration of 1 mM was required for optimal activity. The putative β-glucosidase had an apparent K_m value of 0.19 mM, a V_{max} value of 4.75 U/mg and a k_{cat} value of 316.7/min under the optimal reaction conditions. The biochemical characterization of Bgl1C has enlarged our understanding of the novel enzymes that can be isolated from the soil metagenome.

***Keywords*:** uncultured soil microorganisms, function-based screening strategy, β-glucosidase

It is now widely accepted that the soil metagenome is an important source of many useful biocatalysts (Kowalchuk *et al*., 2007). Recent studies have shown that one gram of soil may contain several thousand different species of microorganisms (Torsvik *et al.*, 1990; Chatzinotas *et al*., 1998; Sessitsch *et al*., 2006). However, 99% of the bacteria in soil cannot be cultured with conventional methods, leaving a large fraction of the soil microbial population unavailable for use (Xu, 2006). Recently, a new strategy that involves the cloning of the total microbial genome, or "metagenome", directly from natural environments into a cultivable bacterium, such as *Escherichia coli*, has been developed (Handelsman, 2004). This approach does not require *in vitro* culturing of microorganisms from environmental samples, thus avoiding the steps for enrichment of dominant microorganisms under selective conditions (Morris, 2006). Previous studies using this strategy have identified a large pool of genes for enzymes (Dubey *et al*., 2006) such as lipase, esterase, hydrolase, amylase, alcohol/aldehyde dehydrogenase, and complex gene clusters encoding enzymes for producing antibiotics. The key advantage of this strategy is that genomes can be obtained from the whole population of microorganisms in a sample rather than from a specific organism in culture. This strategy also provides comprehensive information about the microbes in environmental samples (Fierer *et al*., 2007).

β-Glucosidase (β-D-glucoside glucohydrolase, EC3.2.1.21) catalyzes the hydrolysis of β-glucosidic linkages of various oligosaccharides and glycosides to form glucose and a shorter/debranched oligosaccharide. It is a key rate-limiting enzyme for the cellulose-hydrolyzing system in bacteria and fungi (Bhatia *et al*., 2002). Glucose can be easily fermented into useful chemicals, such as ethanol, which can be used as an environmentally friendly biofuel. β-Glucosidase is present in a broad range of organisms, including microbes, plants, and animals (Healy *et al*., 1995). Recently, many studies have been conducted to isolate novel β-glucosidases from culturable microorganisms (Bhatia *et al*., 2002). Isolation and characterization of β-glucosidases from unculturable microorganisms could provide more information about cellulolytic diversity, and useful enzymes for industrial applications. However, few studies have been carried out on metagenome-derived β-glucosidases (Healy *et al*., 1995; Walter *et al*., 2005; Voget *et al*., 2006; Feng *et al*., 2007; Nam *et al.*, 2008).

To increase our knowledge of β-glucosidases, we screened a metagenomic library from alkaline polluted soil samples, isolated a novel β-glucosidase gene (*bgl1C*), and overexpressed this gene in *E. coli*. To our knowledge, this is the first member of a novel family of β-glucosidase genes that share no similarity with any other known β-glucosidase genes.

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Materials and Methods

DNA manipulation and protein analysis

All DNA manipulations, including cloning and subcloning, transformation of *E. coli* cells, and PCR were performed according to standard techniques (Sambrook and Russell, 2001) or following the manufacturer's instructions unless indicated otherwise. Protein preparation and analysis including protein extraction from *E. coli*, protein quantification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described in standard protocols (Laemmli, 1970).

Screening the metagenome library for β-glucosidase genes

A metagenomic plasmid library was generated in *E. coli* using DNA extracted from alkaline polluted soil in southern China (Jiang and Wu, 2007). The soil metagenomic library was constructed with *E. coli* DH5α as the host and pGEM-3Zf (+) as the cloning vector. The library was stored at -80°C until screening. Colonies of the library were replica plated onto Luria-Bertani (LB) agar plates supplemented with ampicillin (100 μg/ml). Esculin hydrate and ferric ammonium citrate (Sigma, USA) were used to detect β-glucosidase activity according to the method described by Eberhart *et al*. (1964). The plasmids of the positive clones were retransformed into *E. coli* DH5α to confirm that the activities were due to the cloned DNA.

DNA sequence analysis, database search, and gene structure characterization

DNA sequence analysis was performed with the BigDye Terminator Cycle sequencing kit on an ABI Prism 3700 DNA analyzer (Applied Biosystems, USA) using a primer walking strategy. The walking-primers were: SP6; ATTTAG GTGACACTATAG, P1; 5'-TTTAAGATTGGATGGAAGAA C-3', P2; 5'-TGGCAGATGTAACATCTTATA-3', P3; 5'-AGT ATATATAACTCTGAAAGAC-3', P4; 5'-TGGAGTCTACTG ATTTTGAAAT-3', and T7; TAATACGACTCACTATAGGG. Amino acid sequences were deduced with the web-based tool at Expasy (http://www.expasy.org/tools/dna.html). Sequence similarity searches were performed with the BLAST 2.0 program. Amino acid sequence alignment of the target putative protein with homologous proteins was performed with the Align X program, a component of the Vector NTI suite (Informax, USA) using the blosum62mt2 scoring matrix.

Overexpression and purification of the recombinant β-glucosidase protein

The *bgl1C* gene was amplified from the plasmid (pGEXC 2566) isolated from a β-glucosidase producing clone. PCR was carried out in a total volume of 50 μl containing 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM each dNTP, 0.4 μM each primer, 1.0 unit Vent DNA polymerase (NEB, USA) and 10 ng plasmid template. Based on the *bgl1C* gene (1,443 bp) sequences, restriction enzyme sites (underlined) for *Bam*HI and *Hin*dIII were designed in the forward primer/reverse primers; 5'-CGCGGATCCATG TTTGAAGTGAGCATGAA-3'/5'-CGGAAGCTTAAACTTT TTCACTTTACATAGG-3'. The PCR cycling consisted of a denaturation step (96°C for 2 min) followed by 30 cycles of 94°C for 40 sec, 50°C for 30 sec, and 72°C for 2 min, and a final extension at 72°C for 10 min. After amplification, the PCR product was digested with *Bam*HI and *Hin*dIII and ligated into the pETBlue-2 (Novagen, USA) expression vector digested with the same enzymes. The resulting plasmid was transformed into NovaBlue (Novagen) competent cells and subsequently transformed into *E. coli* Tuner (DE3) pLacI (Novagen) to express the target protein.

The transformed bacterial cells were cultured in LB medium containing carbenicillin (50 μg/ml) and chloramphenicol (100 μg/ml) at 37°C, and protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the OD at 660 nm of the bacterial culture reached 0.6. After incubation for an additional 6 h, the cells were harvested, washed twice with phosphate-buffered saline (pH 7.6), and lysed by sonication in 10 ml of 20 mM Tris-HCl, pH 8.0. The lysate was centrifuged twice at 30,000×g for 20 min at 4°C and 1 ml of the supernatant was diluted with 5 ml of column buffer (20 mM Tris-HCl; pH 8.0, 10 mM imidazole, 300 mM NaCl) and applied onto an equilibrated nickel nitrilotriacetic acid (Ni-NTA) column containing 1 ml of Ni-NTA-agarose (Novagen). After a wash with 5 ml of column buffer containing 10 mM imidazole, the recombinant protein was eluted with the same buffer but containing 250 mM imidazole. The target fraction (about 600 μl) was collected. 1,4-Dithiothreitol (DTT) (Promega) was added to a final concentration of 5 mM to the protein solution immediately after its elution from the column. The isolated protein was further purified by gel filtration. A 25 μl aliquot of the Ni-NTA eluate was loaded onto a Superdex 200 PC 3.2/30 column equilibrated with the running buffer (20 mM Tris-HCl; pH 8.0, 200 mM NaCl) and eluted at a flow rate of 40 μl/min. The Superdex 200 PC 3.2/30 column and the standard proteins used for calibration were obtained from Amersham Pharmacia Biotech (USA). The protein concentration was determined by a Bio-Rad protein assay kit with bovine serum albumin as the standard. The protein purified with the Ni-NTA column and gel filtration was used for enzyme activity assays.

Identification of the hydrolysis product

The hydrolysis product of the putative β-glucosidase was identified by high performance liquid chromatography (HPLC). D-Glucosyl-β-(1-4)-D-glucose (Sigma) was used as the substrate for characterization of the β-glucosidase. The enzymatic reaction mixture contained 10 mM D-glucosyl-β-(1-4)-D-glucose, 0.2 M Na_2HPO_4-citric acid buffer, pH 7.0, 1.0 mM $MgCl_2$, and approximately 20 μg purified recombinant protein. The reaction was conducted in a total volume of 2.0 ml at 35°C for 15 min. Upon termination of the reaction with 2.0 ml 1 M Na_2CO_3, the residual protein was removed by centrifugation through a membrane (Vivaspin 500, Vivascience, USA). The filtered reaction sample was separated on a Fast Carbohydrate Cartridge Column (150×4.6 mm, Waters, USA) eluted with double distilled H_2O and acetonitrile (15:85, v/v) as the mobile phase at a flow rate of 1.0 ml/min and detected using a Waters R-401 differential refractometer (Chir *et al.*, 2002; Kim *et al*., 2007).

Physico-chemical characterization of recombinant β-glucosidase protein

Unless otherwise specified β-glucosidase activity was assayed at 40°C by incubating 20 μl of suitably diluted enzyme with 480 μl of 2 mM *p*-nitrophenyl-β-D-glucoside (Sigma) in 50 mM Na-phosphate buffer (pH 7.0) for 15 min. The reaction was stopped by adding 600 μl of 0.4 M Na_2CO_3 and the *p*-nitrophenol (*p*NP) released was determined by reading the absorbance at 420 nm. One unit of β-glucosidase activity (U) is defined as the amount of enzyme that will hydrolyze 1 μmol *p*NP-β-D-glucoside per minute under the experimental conditions described above (Odoux *et al*., 2003). Substrate analogues (2 mM) (Sigma) *p*NP-β-D-cellobioside, *p*NP-α-D-glucoside, *p*NP-β-D-xyloside, *p*NP-β-D-galactoside, methylumbelliferyl-β-D-glucoside, and native oligo- (2 mM) (Sigma) D-glucosyl-β-(1-4)-D-glucose, maltose, lactose, salicin were used to assess the substrate specificity of the purified Bgl1C protein. Glucose released by enzyme hydrolysis of natural glycosides and synthetic aryl-glycosides was measured using the Glucose Oxidase Peroxidase kit from Boehringer Mannheim. The profiles of activity versus pH and activity versus temperature were determined with the standard assay method. To measure the effect of pH on the activity of Bgl1C protein, the enzyme activity was assayed in the range of pH 4.5~9.0 (50 mM Na-phosphate buffer) and pH 8.6~10.0 (0.1 M glycine-NaOH buffer). For the effect of temperature on activity of Bgl1C protein, the enzyme was assayed at various temperatures (20 to 60°C) for 15 min with *p*NP-β-D-glucoside as the substrate in 50 mM Na-phosphate buffer (pH 8.0). Thermostability was determined by pre-incubating the purified enzyme for various intervals up to 180 min at temperatures ranging from 28 to 45°C in 50 mM Na-phosphate buffer (pH 8.0) and analyzing the residual activity with *p*NP-β-D-glucoside as the substrate. Various metal compounds (NaCl, KCl, $MgCl_2$, $ZnCl_2$, $CuCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_2$, $FeCl_3$), chelating agent (ethylenediaminetetraacetic acid, EDTA), and surfactant (SDS) were added to optimal reaction systems to investigate their effects on enzyme activity. The concentrations of metal ions, EDTA, and SDS used were 1 mM, 2 mM, and 1% (v/v), respectively.

Enzyme kinetic assays

Enzyme kinetic parameters of Bgl1C were obtained by measuring the rate of hydrolysis of *pNP-β*-D-glucoside at various concentrations (1~10 mM) at 42°C for 15 min in 50 mM Na-phosphate buffer (pH 8.0). The enzyme concentration was 15 μM and the enzymatic kinetic parameters, K_m and V_{max}, were determined from the Lineweaver-Burk plots using the Enzyme Kinetics computer program (Stanislawski, 1991).

Nucleotide sequence accession number

The *bgl1C* nucleotide sequence has been deposited in the GenBank under accession number EU678637.

Results

Cloning and sequence analysis of a novel β-glucosidase gene

In our previous study, we constructed a plasmid library containing approximately 30,000 clones from metagenomic DNA isolated from alkaline polluted soil (Jiang and Wu, 2007). Plasmid DNA from randomly picked clones was digested with *Eco*RI; the insert DNA was 1 to 15 kb, with an average of approximately 3.5 kb. In this study, from this metagenomic DNA library a clone, named pGEXC2566, was isolated by using the function-based strategy that expressed strong β-glucosidase activity. As shown in Supplementary data Fig. 1S, a black spot surrounded the positive clone pGEXC2566 against a plain background after 24 h at 37°C. The plasmid from this positive clone was extracted and sequenced by primer-walking. The insert DNA sequence (3,627 bp) did not share homology with any other known genes encoding β-glucosidase according to the BLASTx program. Based on the sequence similarity results, we considered that the cloned gene on pGEXC2566 is a novel β-glucosidase gene and named it *bgl1C*. The nucleotide sequence of the putative β-glucosidase gene *bgl1C* and the deduced amino acid sequence of its protein are presented in Supplementary data Fig. 2S. The gene had an open reading frame of 1,443 bp and the deduced peptide was predicted to have a molecular mass of ~57.8 kDa and an isoelectric point of 5.16. When the deduced amino acid sequence of Bgl1C was searched against the NCBI and Expasy databases, amino acid sequence comparison revealed that the deduced Bgl1C peptide had some similarities with hypothetical proteins from *Plasmodium* species and *Borrelia burgdorferi* 118a. These hypothetical proteins include PC000001.00.0 (GenBank accession no. CAH74252) from *Plasmodium chabaudi* (21% identical and 40% similar), reticulocyte binding-like protein 3 (GenBank accession no. AAO38040) from *Plasmodium reichenowi* (22% identical and 43% similar), PF14_0326 (GenBank accession no. XP_001348500) from *Plasmodium falciparum* 3D7 (21% identical and 39% similar), and Bbur9_01505 (GenBank accession no. ZP_02724941) from *Borrelia burgdorferi* 94a (21% identical and 40% similar).

Overexpression and purification of recombinant Bgl1C protein

In order to characterize the function of Bgl1C, we subcloned the gene in frame with a six-histidine tag sequence into expression vector pETBlue-2 and expressed it in *E. coli* Tuner (DE3) pLacI. Initial analysis with crude cell lysates showed that the bacteria containing recombinant plasmid pETBlue-2-*bgl1C* produced a substantial amount of the expected recombinant protein, while this protein was not detectable in cultures of the bacteria containing the empty vector pETBlue-2. The recombinant Bgl1C protein was purified with Ni-NTA Magnetic Agarose Chromatography followed by the removal of degraded and/or nonspecifically bound polypeptides with gel filtration chromatography (GFC). As shown in Supplementary data Fig. 3S, the recombinant Bgl1C protein was purified to homogeneity. The recombinant protein eluted from the column has a molecular weight of ~60 kDa, which is consistent with the theoretical mass of 61 kDa.

Functional characterization of recombinant Bgl1C protein

We determined the product of enzymatic cellobiose hydrolysis by HPLC. The enzymatic product peak eluted earlier than

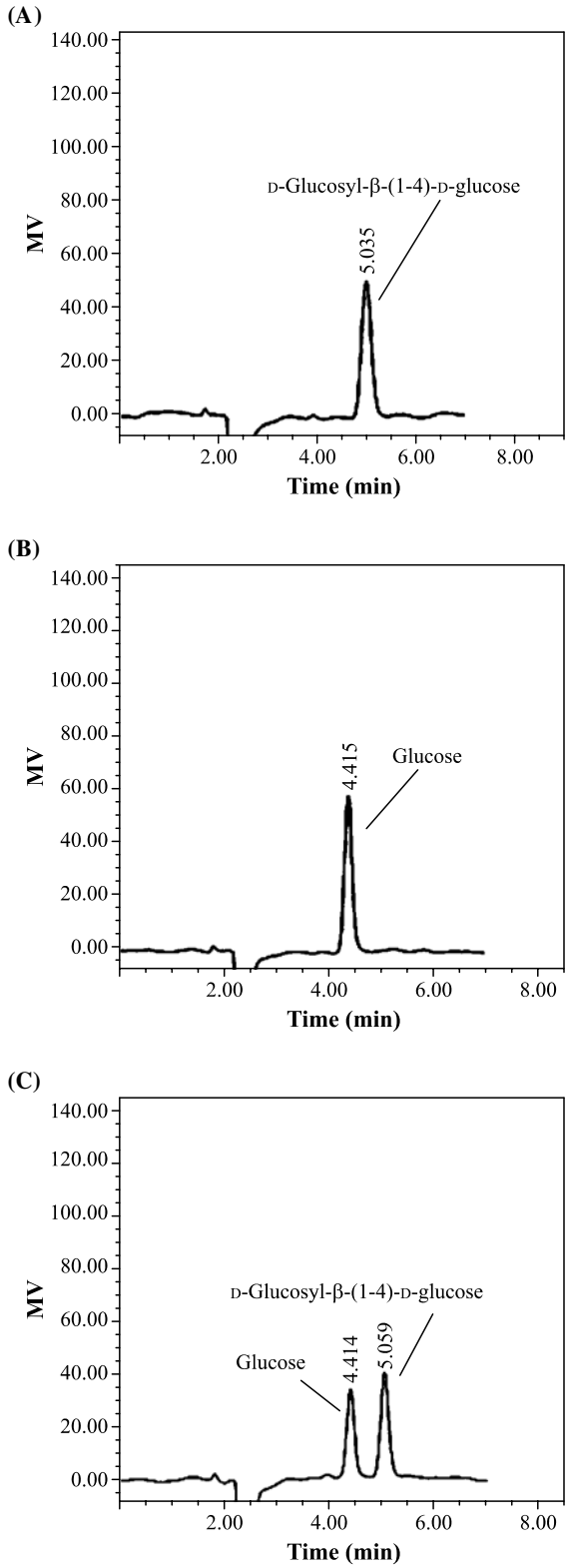

Fig. 1. The reaction product of Bgl1C with D-glucosyl-β-(1-4)-D-glucose as the substrate analyzed by HPLC. (A) HPLC chromatograph of D-glucosyl-β-(1-4)-D-glucose without Bgl1C. (B) HPLC chromatograph of authentic glucose solutions. (C) HPLC chromatograph of the hydrolyzate of D-glucosyl-β-(1-4)-D-glucose catalyzed by Bgl1C protein.

the substrate peak [D-Glucosyl-β-(1-4)-D-glucose], matched the retention time of a glucose standard (Fig. 1). These results suggested that Bgl1C protein catalyzed the formation of glucose from D-glucosyl-β-(1-4)-D-glucose.

Physico-chemical characterization of recombinant Bgl1C protein

To determine the optimal pH for this putative β-glucosidase, we measured the enzymatic activity of the purified Bgl1C protein at various pH values from pH 4.0 to 9.5 using *p*NP-β-D-glucoside as the substrate. The data, presented in Fig. 2A, demonstrated that Bgl1C protein exhibited >80% of its maximal activity in the pH range of 6.0 to 8.5, and the highest activity appeared around pH 8.0. To determine the optimal temperature for the enzymatic reaction of Bgl1C, its activity was measured at pH 8.0 in the temperature range from 20 to 60℃. As shown in Fig. 2B, at a temperature lower than 20℃, the enzyme showed 30% of its maximum activity. Bgl1C exhibited >60% of its maximum activity from 37 to 45℃, and the enzyme reached its highest activity at approximately 42℃. To study its thermostability, we incubated the purified putative β-glucosidase for 5 to 180 min in a water bath at different temperatures and measured its residual activity under the standard assay conditions. Fig. 3

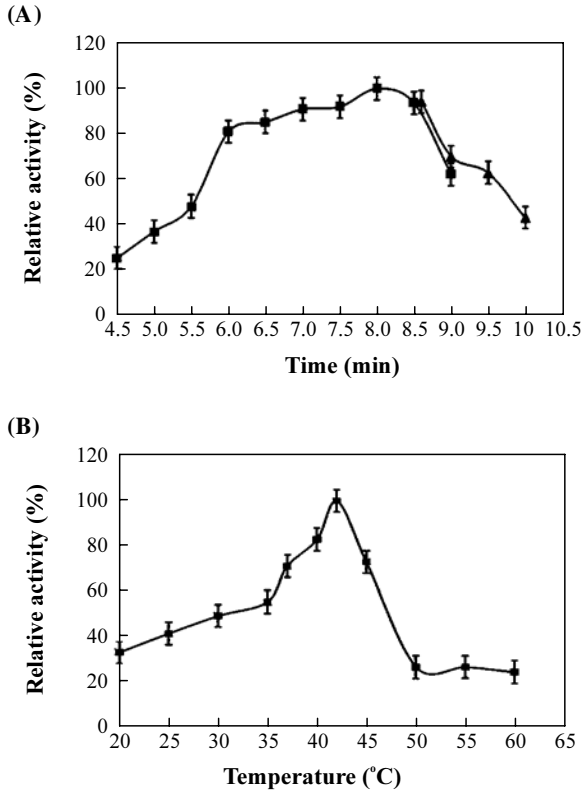

Fig. 2. (A) Effects of pH on the enzymatic activity of Bgl1C protein; the buffers used were Na-phosphate buffer (■) (pH 4.5~9.0), glycine-NaOH buffer (▲) (pH 8.6~10.0). The relative activities are the enzyme activities at each pH divided by the maximal activity. (B) Effects of temperature on the enzymatic activity (■) of Bgl1C protein. The relative activities are the enzyme activities at each temperature divided by the maximal activity.

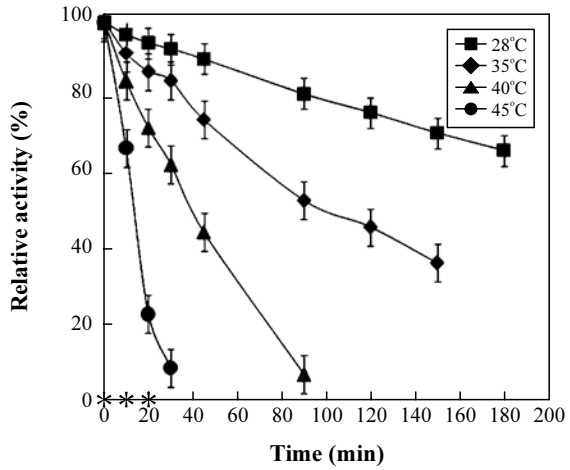

Fig. 3. The thermostability of Bgl1C protein. Bgl1C was incubated at 28°C (■), 35°C (◆), 40°C (▲), 45°C (●) for the indicated time. The activity at time 0 was defined as 100%.

shows that the activity of Bgl1C decreased quickly with increasing temperature in the absence of any stabilizer. When the temperature was higher than 45°C, Bgl1C protein lost a majority of its activity within 20 min. The effects of metal ions, EDTA, and SDS on the hydrolytic activity of Bgl1C protein were determined with *p*NP-β-D-glucoside as the substrate by measuring the residual activity. The enzyme activity of Bgl1C without added metal ions was taken as 100%. The enzyme activity was slightly reduced by 1 mM concentrations of cations such as $CuCl_2$ (90%), $ZnCl_2$ (88%), and $MgCl_2$ (93%). In the presence of 1 mM $MnCl_2$, $FeCl_2$, and $FeCl_3$ the residual activity decreased dramatically by 37%, 46%, and 61%, respectively. The chelating agent EDTA and the anionic surfactant SDS reduced the activity to 63% and 50%, respectively. In contrast, the activity of the recombinant enzyme was stimulated slightly by 1 mM NaCl (108%). Bgl1C protein was stimulated significantly by the presence of 1 mM $CaCl_2$, to 139% of its original activity, but the activity was inhibited beyond 2.0 mM (data not shown). These results suggested that a $CaCl_2$ concentration of 1 mM was required for the optimal activity of Bgl1C protein. The enzyme activity reached 3.36 U/mg protein using *p*NP-β-D-glucoside as the substrate under the optimal reaction conditions (Supplementary data Table 1S).

Substrate specificity and enzyme kinetics

Relative rates of hydrolysis of various substrates by the purified Bgl1C protein were measured (Table 1). The enzyme could hydrolyze *p*NP-β-D-glucoside and D-glucosyl-β-(1-4)-D-glucose effectively. The synthetic chromogenic substrates methylumbelliferyl-β-D-glucoside and *p*NP-β-D-cellobioside were hydrolyzed at 42% and 34% of the rate for *p*NP-β-D-glucoside, respectively. The recombinant enzyme was also active with salicin (36%), a natural coumaric substrate. The purified recombinant enzyme had very little (<5%) or no activity on lactose and maltose. It also had no or very little activity on *p*NP-β-D-lactoside, *p*NP-β-D-galactoside, and *p*NP-α-D-glucoside (Table 1). These results indicate that Bgl1C protein is a specific β-glucosidase. The kinetic parameters of the recombinant enzyme Bgl1C protein were determined using different *p*NP-β-D-glucoside concentrations as the substrate. The initial rate of the enzyme reaction was measured under the optimal reaction conditions. The reaction kinetic parameters of the purified enzyme were determined from double reciprocal Lineweaver-Burk plots. The putative β-glucosidase has an apparent K_m value of 0.19 mM, a V_{max} value of 4.75 U/mg, a k_{cat} value of 316.7/min and a k_{cat}/K_m value of 1667/mM/min.

Table 1. Substrate specificity of recombinant Bgl1C protein, Activity on *p*NP-β-D-glucoside was taken as 100%, which corresponds to a specific activity of 3.36 U/mg

Substrate	Relative activity* (%)
*p*NP-β-D-glucoside	100
*p*NP-β-D-xyloside	0
*p*NP-β-D-galactoside	<5
*p*NP-β-D-cellobioside	34
*p*NP-α-D-glucoside	0
Methylumbelliferyl-β-D-glucoside	42
D-Glucosyl-β-(1-4)-D-glucose	72
Maltose	0
Lactose	<5
Salicin	36

Discussion

The approach of generating a metagenome library from soils or sediments that are known to harbor a high level of microbial diversity has been used successfully to find a wide variety of novel catalysis and secondary metabolites (Handelsman, 2004; Dubey *et al*., 2006). It is also interesting to find some novel enzymes, with no homology to the corresponding protein family. However, few studies have been carried out on β-glucosidase-like activity. In 2005, only one putative β-glucosidase from *Pichia etchellsii* was identified and showed no homology with known β-glucosidases. In contrast, this protein shares a high degree of identity with several cell-surface-associated proteins (Roy *et al*., 2005). Here, we isolated a novel gene (*bgl1C*) encoding a putative protein with β-glucosidase activity from uncultured microorganisms in alkaline polluted soils. As Bgl1C is a metagenome-derived β-glucosidase, it is not easy to determine from which microorganism it came. Based on multiple amino acid sequence alignments, the deduced Bgl1C sequence did not match any β-glucosidase. Our results suggest that this enzyme may represent a new family of glycoside hydrolase proteins. Studies of this protein will provide new insights into protein structure and catalytic mechanisms, and also allow functional assignments for some proteins currently designated as "hypothetical" or "conserved hypothetical" proteins in the databases (Ferrer *et al*., 2005).

Identification of a novel β-glucosidase-like gene from uncultured microorganisms demonstrated the advantage of a metagenomic library for cloning novel genes through function-based screening with *E. coli* as the host. This appears to be the first study on the cloning and the characterization

Table 2. Properties of β-glucosidases from various sources. Kinetics parameters of β-glucosidases were shown for *p*NP-β-D-glucoside

Source	Mr (kDa)	K_m (mM)	Opt. pH	Opt. temp. (°C)	V_{max} (U/mg)	Reference
Aspergillus niger	330	0.18	4.6-5.3	70	NR	Rashid and Siddqui (1997)
Talaromyces emersonii	90.59	0.13	4.02	71.5	512	Murray *et al.* (2004)
Ceriporiopsis subvermispora	110	3.3	3.5	60	0.13	Magalhaes *et al.* (2006)
Piptoporus betulinus	36	1.8	4.0	60	19	Valaskova and Baldria (2006)
Daldinia eschscholzii	64	1.5	5.0	50	3.2	Karnchanatat *et al.* (2007)
Fomitopsis pinicola	105	1.76	4.5	50	1710	Joo *et al.* (2009)
Uncultured microorganisms	55	0.16	6.5	55	19.10	Kim *et al.* (2007)
Uncultured microorganisms	57.8	0.19	8.0	42	4.75	This work

NR: not reported.

of a putative β-glucosidase gene from microbes in alkaline polluted soils. The hitherto known β-glucosidases, with the exception of glycoside ceramidase (acid β-glucosidase), which is placed in family 30, are placed in are placed in family 1, family 3 or family 9 of glycoside hydrolases (http://www.cazy.org/fam/acc_GH.html). Family 1 comprises more than 1400 β-glucosidases from archaea, bacteria, and eukaryota and also includes 6-phosphoglycosidases and thioglucosidases. Most family 1 enzymes also show significant β-galactosidase activity. Family 3 of glycoside hydrolases consists of more than 1500 β-glucosidases and hexosaminidases of bacterial, mold, and yeast origin. A majority of family 9 enzymes come from bacteria and eukaryote. In the present study, the putative β-glucosidase of Bgl1C protein showed very little activity on *p*NP-β-D-galactoside (<5%), suggested that Bgl1C was more similar to the biochemical characterization of enzymes from glycoside hydrolase family 3 than family 1. As enzymes are known to display habitat-related characteristics, metagenome screening is an excellent strategy to obtain novel natural biocatalysts with unusual properties. We investigated the optimal temperature and pH for the hydrolysis reaction of *p*NP-β-D-glucoside by Bgl1C protein. It's interesting to find that the enzyme maintains activity over a broad pH range from 6.0 to 9.0 because the optimal activities of purified β-glucosidases from various microbial sources range from 3 to 7 (Bhatia *et al*., 2002). The results of initial ion studies with Bgl1C, particularly inhibition by $MnCl_2$, $FeCl_2$, and $FeCl_3$ and a significant effect of EDTA treatment, differ from those of other glycoside hydrolase family 3 β-glucosidases (Hashimoto *et al*., 1998). This could be environmentally relevant since Bgl1C is a metagenome-derived protein. Table 2 shows a comparison of the properties of various β-glucosidases from a number of different sources. Bgl1C protein had a comparable V_{max} value of 3.36 U/mg for *p*NP-β-D-glucoside. In comparison, *p*NP-β-D-glucoside V_{max} values of purified β-glucosidases from other sources ranged from 0.13 to 1710 (Table 2). Thus, the K_m value for Bgl1C protein was in agreement with this recorded for β-glucosidase from uncultured microorganisms (Kim *et al.*, 2007) and *Aspergillus niger* (Rashid and Siddqui, 1997). The HPLC result demonstrated that the recombinant Bgl1C protein hydrolyzed D-glucosyl-β-(1-4)-D-glucose to glucose. The functional characterization of Bgl1C may provide new insight into the relationship between the sequence, structure, and activity of the known β-glucosidase protein family. Further studies of directed evolution and three-dimensional structures to elucidate the catalyzing mechanism will deepen our understanding of Bgl1C and other β-glucosidases in general.

In conclusion, we have identified a novel gene (*bgl1C*) encoding an enzyme with β-glucosidase-like activity by function-based screening of a metagenomic library from uncultured microorganisms. Furthermore, we performed a detailed biochemical characterization of this recombinant putative β-glucosidase, including molecular characterization, specific activity, pH-activity profile, metal ion-activity profile and substrate specificity. A more detailed biochemical characterization of Bgl1C is currently in progress. These results are a first step toward a better understanding of the properties of Bgl1C protein.

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