Cloning and Identification of Essential Residues for Thermostable β-Glucosidase (BglB) from *Thermotoga maritima*

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A hyperthermophilic bacterium *Thermotoga maritima* produced thermostable β-glucosidase. The gene encoding β-glucosidase from *T. maritima* MSB8 was cloned and expressed in *Escherichia coli*. The enzyme (BglB) hydrolyzed β-glucosidic linkages between glucose and alkyl, aryl of saccharide groups such as salicin, arbutin, and pNPG. The insert DNA contained ORF with 2,166 bp encodes a 721 amino acids (calculated molecular mass of 80,964 and pI of 4.93). The amino acid sequence of BglB showed the similarity to family 3 glycosyl hydrolases. The molecular weight of the enzyme was estimated to be approximately 81 kDa by MUG-nondenaturing PAGE (4-methylumbelliferyl β-D-glucoside-nondenaturing polyacrylamide gel electophoresis) and SDS-PAGE. The β-glucosidase exhibited maximal activity at pH 7.0 and 80°C. By exchanging two possible residues (Glu-232 and Asp-242) to Ala by site-directed mutagenesis method, it was found that these were essential for enzymatic activity.

Key words – *Thermotoga maritima*, β-glucosidase, essential residue, site-directed mutagenesis

Introduction

Thermotoga maritima, a marine hyperthermophile isolated from geothermally heated biotopes[45], can be grown in temperatures of up to 90°C with an optimal temperature of around 80°C[9]. *T. maritima* sharing with other *Thermotogales*, specifically *T. neapolitana*, has both the capacity to catabolize a wide variety of α- and β-linked glucans and several polysaccharides[44]. In the last few years, a variety of saccharolytic enzymes have been isolated from *Thermotoga* species, such as cellobiohydrolases[30,39], amylases[2,25], xylanases[19,31,35,46], endoglucanases[4-8], β-glucosidases [13-15,47]. All of these enzymes are thermophilic and resistant to high temperatures, denaturing agents, solvents, and proteolytic enzymes[11]. Among them, β-glucosidases broadly hydrolyze shorter oligosaccharides to glucose for use in a novel Embden-Meyerhof pathway (EMP)[1]. *T. maritima* uses glucose, in contrast to the archaea, primarily by the conventional form of EMP and the phosphorylated Entner-Doudoroff pathway (EDP)[17,33,34]. Previous studies reported that *T. maritima* has two different intracellular β-glucosidases, BglA and BglB, these Bgl proteins already sequenced, and the deduced amino acid sequence shown belongs to glycosyl hydrolase family 1 and 3, respectively[15].

In the study of β-glucosidase (Abg) of *Agrobacterium faecalis*, Wang *et al.*[42] describes that two active site amino acid residues play key roles in catalytic activity of enzyme. One acts as a nucleophile attacking the anomeric center region of the substrate and other residue serves as a general acid/base catalyst providing protonic assistance between enzyme and substrate. In all cases, these catalytic groups have the carboxylic side chains of glutamic or aspartic acid residues[36]. Therefore, the role of acid/base catalyst can be identified by searching for conserved glutamic or aspartic acid residues through sequence alignments of related enzymes[42].

Here we report a site-directed mutagenesis study of conserved catalytic residues (Glu-232 and Asp-242) for enzymatic activity and a characterization using MUG-nondenaturing PAGE of β-glucosidase (BglB) from *T. maritima* in *E. coli*.

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Materials and Methods

Bacterial strains and growth conditions

E. coli DH5α, BL21 (DE3), and recombinant *E. coli* cells

harboring *bgl*B gene were cultured in Luria-Bertani (LB) medium or LB medium containing the appropriate antibiotics, ampicillin (50 μg/ml)[22,27]. Genomic DNA of *T. maritima* MSB8 was provided from Dr. Karl O. Stetter (Regensburg, Germany)[26].

Recombinant DNA techniques

Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning related techniques were followed as described by Sambrook and Russell[32]. Restriction enzymes and DNA modifying enzymes were purchased from Promega (Madison, Wisconsin, USA) and gel extraction kit and plasmid DNA isolation kit were purchased from iNtRON Biotechnology (Seongnam, Korea). Other chemicals were purchased from Sigma Chemical (St. Louis, Missouri, USA).

DNA sequencing

Nucleotide sequences were determined with the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CT, USA). The samples were analyzed with an automated DNA sequencer (Model 3100; Applied Biosystems, Foster City, CA, USA). The BLAST program was used to find the protein coding regions.

Cloning of the β-glucosidase gene

To amplify *bgl*B from the *T. maritima* MSB8 chromosome, specific oligonucleotide primers were designed based on the database. The sense and antisense specific oligonucleotide primers are 5′-ATCCTCTGCGGGTGGGTCTA-3′ (forward) and 5′-CAAGCTCCTCGTCGGTAGCA-3′ (reverse), respectively. The amplified product of approximately 2.9 kb was purified from an agarose gel using gel extraction kit, PCR product was cloned into pGEM-T Easy vector (Promega, WI, USA) and was transformed into *E. coli*. For the high expression of *bgl*B, the PCR product with primers, 5′-GGATCCATGGAAAGGATCGATGAAA-3′ (forward, containing a *Bam*HI site as underlined) and 5′-AAGCTTGTGGTTTGAATCTCTTCTC-3′ (reverse, containing a *Hin*dIII restriction site as underlined) was cloned into expression vector pET-21a(+) (Novagen, USA) using *Bam*HI and *Hin*dIII sites, resulting in the addition of C-terminal $(His)_6$ tag. The resulted vector was designated as pET-21a(+)/BglB (pTM0026). The absence of additional mutation within the coding region of BglB was verified by DNA sequencing with the database sequences for *T. maritima*.

Site-directed mutagenesis

The *bgl*B gene encoding the β-glucosidase was used for site-directed *in vitro* mutations. Site-directed *in vitro* mutations were performed by using synthetic oligonucleotide primers for E232A (pET0027) and D242A (pET0028). The sequences of primer is as follows; 5′-CAGGGAAGAATGG GCATTTGACGGTTTC-3′ (forward) and 5′-GAAACCGTCA AATGCCCATTCTTCCCTG-3′ (reverse) for E232A, 5′-TTTC GTGATGAGCGCCTGGTACGCGGG-3′ (forward) and 5′-C CCGCGTACCAGGCGCTCATCACGAAA-3′ (reverse) for D242A. The 50 μl of reaction mixtures were contained 1 μl of the pTM0026 DNA (80 ng/μl), 10 pmol of each primer, 5 μl of 2 mM dNTP mixture, 5 μl of 10x Pfu DNA polymerase buffer containing 20 mM $MgSO_4$, and 2.5 U of cloned *Pfu* DNA polymerase purchased from Stratagene (La Jolla, CA, USA). PCR products were incubated on ice for 5 min, and added 1 μl of *Dpn*I restriction enzyme (10 U/μl), incubated at 37°C for 1 hr. Following *in vitro* mutation, the *Dpn*I treated plasmids were transformed into *E. coli* DH5α according to the manufacturer's specifications of site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

Enzyme assay

β-Glucosidase acitivity was determined using p-nitrophenyl β-D-glucopyranoside (pNPG), 4-hydroxyphenyl β-D-glucopyranoside (arbutin), 2-(hydroxymethyl) phenyl β-D-glucopyranoside (salicin), as aryl-β-D-glucoside substrates, and cellobiose. The enzymatic activity of the β-glucosidase was mainly determined by measuring p-nitrophenol released from pNPG. The assay mixture, consisting of 5 mM pNPG in 50 mM sodium phosphate buffer (pH 7.0) was incubated with the enzyme for 10 min in a total volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml of 0.2 M glycine-NaOH (pH 10.5), and the amount of p-nitrophenol released was determined by measuring the absorbance at 405 nm. One unit of β-glucosidase is defined as the amount of enzyme required to release 1 mol of p-nitrophenol per minute under the same conditions[19,26]. All assays were carried out in 50 mM (final concentration) sodium phosphate buffer (pH 7.0) at 80°C unless otherwise cited.

In order to detect the β-glucosidase activity for the sali-

cin as substrate, cell suspension was added to 800 μl of 30 mM salicin in 50 mM phosphate buffer (pH 7.0). After 30 min of incubation at 80°C, the enzymatic reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. The production of saligenin from salicin was detected.

The arbutin used to detect the enzymatic activity was measured by washing the culture and resuspending it in 0.8 ml of 50 mM phosphate buffer (pH 7.0). The reaction was started by adding a 0.5 ml of 30 mM arbutin, and was stopped by adding 0.5 ml of 1 M Na_2CO_3. The activity on cellobiose was measured by using the dinitrosalicylic assay. Thermostability data were obtained by pre-incubating β-glucosidase samples in 50 mM sodium phosphate buffer (pH 7.0) at different temperatures and at different intervals of time and measuring residual activity. The effects of various metal ions classified by a concentaration of 10, 20, and 50 mM on β-glucosidase activity were also examined.

Expression and purification of enzyme

All purification steps were performed at ambient temperature (approximately 25°C). The culture of *E. coli* BL21 (DE3) with pET-21a(+)/BglB was grown at 37°C to mid-log phase in LB medium containing 100 μg/ml ampicillin. The transcription was then induced by adding IPTG to a final concentration of 0.5 mM, and growth was continued for 6 hrs. The cells were harvested by centrifugation (6,000 rpm, 10 min) and washed twice with 10 mM Tris-HCl buffer (pH 7.0). The cells were resuspended in the same buffer and stored at -20°C. The frozen cells were mixed with 10 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mg of bovine DNase 1 (Sigma, USA) and incubated at 37°C for 30 min. Triton X-100 was added to the suspension, resulting in a final concentration of 2.5%. Then, the cell suspension was heated at 80°C for 10 min and centrifuged at 5,000 rpm for 20 min. The supernatant was collected and stored at 4°C. The solubilized recombinant BglB with His-tag (His-BglB) was applied on a Ni-NTA column (Qiagen, USA). BglB was eluted with 100 mM imidazole with 0.1% Triton X-100. The fractions with β-glucosidase activity eluted as a single protein peak and the purity of the enzyme were assessed by SDS-PAGE. The protein concentration was determined by the method of Bradford[3].

MUG-native-PAGE and SDS-PAGE of BglB

The nondenaturing-polyacrylamide gel electrophoresis (native-PAGE) and SDS-PAGE were carried out using Bio-Rad Mini-PROTEIN 3 Cell electrophoresis unit (Bio-Rad, USA). Identification of a *bgl*B gene product was performed in 0.75 mm gels in a vertical slab unit by a modification described by Lim *et al.*[25]. The separating gel contained 10% acrylamide and 0.5% bisacrylamide. The purified enzyme mixed with sucrose-dye solution (50% sucrose, 0.1% bromophenol blue) in a ratio of 1:1 (v/v) and electrophoresed at 100 V and 4°C until the tracking dye migrated to the bottom of the gel. Active staining of the β-glucosidase activity in a nondenaturation-PA gel was performed after washing the gel twice in 50 mM sodium-citrate buffer (pH 6.5). The gel was incubated in 50 mM sodium-citrate buffer (pH 6.5) at 80°C for 6 hrs. The acrylamide gels were incubated in 1 mM 4-methylumbelliferyl β-D-glucoside (MUG) (Sigma Chemical Co., USA) for approximately 15 min to 60 min at 37°C with gentle shaking and briefly rinsed with buffer. Fluorescent band could be visualized immediately on a transilluminator (312 nm)[44]. After native-PAGE, active band was eluted from a gel and then electrophoresed by SDS-PAGE.

Results

Cloning and nucleotide sequence of *bglB* gene

The *bgl*B gene was cloned by PCR amplification of the genes from *T. maritima* MSB8 by using the synthetic oligonucleotide primers. The 2.9 kb fragment was ligated to pGEM-T Easy vector and was transformed into *E. coli* DH5 α. The size of inserted DNA and each orientation of restriction cleavage sites were determined (Fig. 1). The insert DNA in pTM0025 contained a restriction sites for *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Hin*dIII, *Nco*I, *Sca*I, and *Xho*I.

The DNA sequence of about 2.9 kb fragment from pTM0025 was determined. Figure 2 shows the *bgl*B structural gene along with its flanking regions. There is an open reading frame (ORF) composed of 2,166 nucleotides encoding a protein of 721 amino acids with a predicted molecular weight of 80,964 Da. The ATG initiation codon at nucleotide position 245 is preceded by a putative Shine-Dalgarno sequence, AGGAGGG. The ORF is ended by TGA stop codon at position 2,410 (Accession number NP_227841).

Comparison of BglB to the other known β-glucosidase

The National Center for Biotechnology Information BLAST e-mail server was used to search the peptide se-

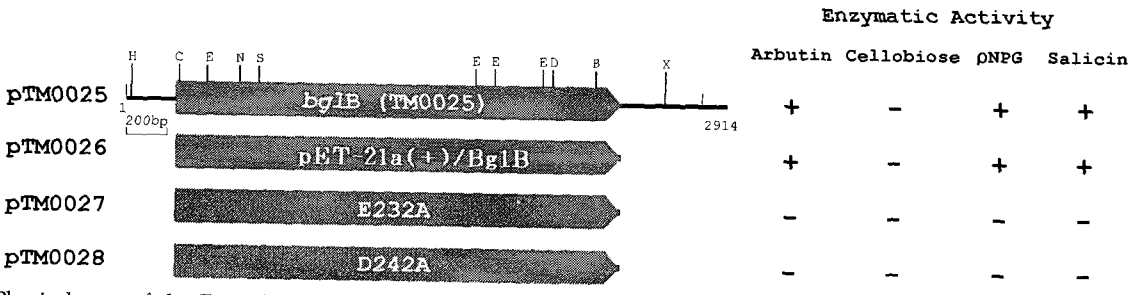

Fig. 1. Physical map of the *T. maritima* β-glucosidase gene. ORF is showed arrow. The cleavage sites of restriction enzymes *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Hin*dIII, *Nco*I, *Sca*I, and *Xho*I are shown. pTM0025 was constructed by cloning a 2.9 kb PCR product of *T. maritima bgl*B into the pGEM-T easy vector. pTM0026 was derived by cloning into pET-21a(+) expression vector. pTM0027 (E232A), pTM0028 (D242A) was derived by point mutation by site-directed mutagenesis.

quence databases for proteins homologous to the β -glucosidase protein sequence. The β-glucosidase encoded by this open reading frame revealed homology to β -glucosidase from variety of organisms, and it is classified in family 3 of the glycosyl hydrolases. A homology matrix based on alignment is present in Table 1. It reveals that the *T. maritima* MSB8 β-glucosidase has about 86.1% *Thermotoga neapolitana* (CAB01407), 47.0% *Prevotella albensis* (CAC07184), 44.0% *Clostridium thermocellum* ATCC 27405 (ZP_00060124), and 35.5% *Bacteriodes thetaiotaomicron* YPI-5482 (AAO78420). The analysis of pairwise similarity of *bgl*B gene suggests that BglB belongs to glycosyl hydrolase family 3.

Purification and characterization of the BglB

Using an *E. coli* strain that overexpressed BglB, the protein was purified using column filtration techniques as described in Materials and Methods. Protein fractions from

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1     CAAGCTCCTCGTCGGTAGCATCCCGAAGCTTTACAGGAAACTGGAGGATCTGTGATGTTTACAAAAGCGGTTCTCTCCATTTTTCTTGGGCTCTGGTTCTTGAATTGATAGTTCTGTTT
121   TATTACCTGTGGCGTGGCTTGAGGCCAGTGGAGTTCTACCTAAATTTAGGACTCCTCGGACTCACAGTACCTTTCCTTGTGTTTCTTGTCGTCAGAGAAAAGAAAAAGAGGAGGGAAGAA
                                                                                                                    SD
241   GACGATGGAAAGGATCGATGAAATTCTCTCTCAGTTAACTACAGAGGAAAAGGTGAAGCTCGTTGTGGGGGTTGGTCTTCCAGGACTTTTTGGGAACCCACATTCCAGAGTGGCGGGTGC
1       M  E  R  I  D  E  I  L  S  Q  L  T  T  E  E  K  V  K  L  V  V  G  V  G  L  P  G  L  F  G  N  P  H  S  R  V  A  G  A
361   GGCTGGAGAAACACATCCCGTTCCAAGACTTGGAATTCCTGCGTTTGTCCTGGCAGATGGTCCCGCAGGACTCAGAATAAATCCCACAAGGGAAAACGATGAAAACACTTACTACACGAC
40      A  G  E  T  H  P  V  P  R  L  G  I  P  A  F  V  L  A  D  G  P  A  G  L  R  I  N  P  T  R  E  N  D  E  N  T  Y  Y  T  T
481   GGCATTTCCCGTTGAAATCATGCTCGCTTCTACCTGGAACAGAGACCTTCTGGAAGAAGTGGGAAAAGCCATGGGAGAAGAAGTTAGGGAATACGGTGTCGATGTGCTTCTTGCACCTGC
80      A  F  P  V  E  I  M  L  A  S  T  W  N  R  D  L  L  E  E  V  G  K  A  M  G  E  E  V  R  E  Y  G  V  D  V  L  L  A  P  A
601   GATGAACATTCACAGAAACCCTCTTTGTGGAAGGAATTTCGAGTACTACTCAGAAGATCCTGTCCTTTCCGGTGAAATGGCTTCAGCCTTTGTCAAGGGAGTTCAATCTCAAGGGGTGGG
120     M  N  I  H  R  N  P  L  C  G  R  N  F  E  Y  Y  S  E  D  P  V  L  S  G  E  M  A  S  A  F  V  K  G  V  Q  S  Q  G  V  G
721   AGCCTGCATAAAACACTTTGTCGCGAACAACCAGGAAACGAACAGGATGGTAGTGGACACGATCGTGTCCGAGCGAGCCCTCAGAGAAATATATCTGAAAGGTTTTGAAATTGCTGTCAA
160     A  C  I  K  H  F  V  A  N  N  Q  E  T  N  R  M  V  V  D  T  I  V  S  E  R  A  L  R  E  I  Y  L  K  G  F  E  I  A  V  K
841   GAAAGCAAGACCCTGGACCGTGATGAGCGCTTACAACAAACTGAATGGAAAATACTGTTCACAGAACGAATGGCTTTTGAAGAAGGTTCTCAGGGAAGAATGGGGATTTGACGGTTTCGT
200     K  A  R  P  W  T  V  M  S  A  Y  N  K  L  N  G  K  Y  C  S  Q  N  E  W  L  L  K  K  V  L  R  E  E  W  G  F  D  G  F  V
                                                                                                                    ▲
961   GATGAGCGACTGGTACGCGGGAGACAACCCTGTAGAACAGCTCAAGGCCGGAAACGATATGATCATGCCTGGGAAAGCGTATCAGGTGAACACAGAAAGAAGATGAAATAGAAGAAAT
240     M  S  D  W  Y  A  G  D  N  P  V  E  Q  L  K  A  G  N  D  M  I  M  P  G  K  A  Y  Q  V  N  T  E  R  R  D  E  I  E  E  I
        ▲
1081  CATGGAGGCGTTGAAGGAGGGAAAATTGAGTGAGGAGGTTCTCGATGAGTGTGTGAGAAACATTCTCAAAGTTCTTGTGAACGCGCCTTCCTTCAAAGGGTACAGGTACTCAAACAAGCC
280     M  E  A  L  K  E  G  K  L  S  E  E  V  L  D  E  C  V  R  N  I  L  K  V  L  V  N  A  P  S  F  K  G  Y  R  Y  S  N  K  P
1201  GGATCTCGAATCTCACGCGGAAGTCGCCTACGAAGCAGGTGCGGAGGGTGTTGTCCTTCTTGAGAACAACGGTGTTCTTCCGTTCGATGAAAATACCCATGTCGCCGTCTTTGGCACCGG
320     D  L  E  S  H  A  E  V  A  Y  E  A  G  A  E  G  V  V  L  L  E  N  N  G  V  L  P  F  D  E  N  T  H  V  A  V  F  G  T  G
1321  TCAAATCGAAACAATAAAGGGAGGAACGGGAAGTGGAGACACCCATCCGAGATACACGATCTCTATCCTTGAAGGCATAAAAGAAAGAAACATGAAGTTCGACGAAGAACTCGCTTCCAC
360     Q  I  E  T  I  K  G  G  T  G  S  G  D  T  H  P  R  Y  T  I  S  I  L  E  G  I  K  E  R  N  M  K  F  D  E  E  L  A  S  T
1441  TTATGAGGAGTACATAAAAAAGATGAGAGAAACAGAGGAATATAAACCCAGAACCGACTCTTGGGGAACGGTCATAAAACCGAAACTCCCAGAGAATTTCCTCTCAGAAAAAGAGATAAA
400     Y  E  E  Y  I  K  K  M  R  E  T  E  E  Y  K  P  R  T  D  S  W  G  T  V  I  K  P  K  L  P  E  N  F  L  S  E  K  E  I  K
1561  GAAAGCTGCAAAGAAAAACGATGTTGCAGTTGTTGTGATCAGTAGGATCTCCGGTGAGGGATACGACAGAAAGCCGGTGAAAGGTGACTTCTACCTCTCCGATGACGAGCTGGAACTCAT
440     K  A  A  K  K  N  D  V  A  V  V  V  I  S  R  I  S  G  E  G  Y  D  R  K  P  V  K  G  D  F  Y  L  S  D  D  E  L  E  L  I
1681  AAAAACCGTCTCGAAAGAATTCCACGATCAGGGTAAGAAAGTTGTGGTTCTTCTGAACATCGGAAGTCCCATCGAAGTCGCAAGCTGGAGAGACCTTGTGGATGGAATTCTTCTCGTCTG
480     K  T  V  S  K  E  F  H  D  Q  G  K  K  V  V  V  L  L  N  I  G  S  P  I  E  V  A  S  W  R  D  L  V  D  G  I  L  L  V  W
1801  GCAGGCGGGACAGGAGATGGGAAGAATAGTGGCCGATGTTCTTGTGGGAAAGATTAATCCCTCCGGAAAACTTCCAACGACCTTCCCGAAGGATTACTCGGACGTTCCATCCTGGACGTT
520     Q  A  G  Q  E  M  G  R  I  V  A  D  V  L  V  G  K  I  N  P  S  G  K  L  P  T  T  F  P  K  D  Y  S  D  V  P  S  W  T  F
1921  CCCAGGAGAGCCAAAGGACAATCCGCAAAGAGTGGTGTACGAGGAAGACATCTACGTGGGATACAGGTACTACGACACCTTCGGTGTGGAACCTGCCTACGAATTCGGCTACGGCCTCTC
560     P  G  E  P  K  D  N  P  Q  R  V  V  Y  E  E  D  I  Y  V  G  Y  R  Y  Y  D  T  F  G  V  E  P  A  Y  E  F  G  Y  G  L  S
2041  TTACACAAAGTTTGAATACAAAGATTTAAAAATCGCTATCGACGGTGAGACGCTCAGAGTGTCGTACACGATCACAAACACTGGGGACAGAGCTGGAAAGGAAGTCTCACAGGTCTACAT
600     Y  T  K  F  E  Y  K  D  L  K  I  A  I  D  G  E  T  L  R  V  S  Y  T  I  T  N  T  G  D  R  A  G  K  E  V  S  Q  V  Y  I
2161  CAAAGCTCCAAAAGGAAAAATAGACAAACCCTTCCAGGAGCTGAAAGCGTTTCACAAAACAAAACTTTTGAACCCGGGTGAATCAGAAGAAATCTCCTTGGAAATTCCTCTCAGAGATCT
640     K  A  P  K  G  K  I  D  K  P  F  Q  E  L  K  A  F  H  K  T  K  L  L  N  P  G  E  S  E  E  I  S  L  E  I  P  L  R  D  L
2281  TGCGAGTTTCGATGGGAAAGAATGGGTTGTCGAGTCAGGAGAATACGAGGTCAGGGTCGGTGCATCTTCGAGGGATATAAGGTTGAGAGATATTTTTCTGGTTGAGGGAGAGAAGAGATT
680     A  S  F  D  G  K  E  W  V  V  E  S  G  E  Y  E  V  R  V  G  A  S  S  R  D  I  R  L  R  D  I  F  L  V  E  G  E  K  R  F
2401  CAAACCATGAAAAGGGGGTAATATGATGAGCAGGCTGGTTTTCGCTCTGCTTCTCTTTCCTGTTTTCATTCTGGCTCAAAACATCCTTGGCAACGCTTCTTTCGATGAACCAATTCTCAT
720     K  P  *
2521  CGCAGGTGTGGATATAGACCCACCCGCAGAGGATGGCTCTATAGACACAGGAGGAAACTGGGTATTCTTCACCAATTCAAACGGTGAGGGAACGGCTCGAGTCGAAAACGGCGTTCTCGT
2641  GGTTGAGATAACAAACGGAGGAGATCACACCTGGTCGGTTCAGATCATACAGGCTCCCATACGTGTTGAGAAACTCCACAAGTACAGAGTTTCTTTCCGAGCCAAGGCTTCCTCTCAAAA
2761  GAACATCGGAAAAGGGGGTAATATGATGAGCAGGCTGGTTTTCGCTCTGCTTCTCTTTCCTGTTTTCATTCTGGCTCAAAACATCCTTGGCAACGCTTCTTTCGATGAACCAATTCTCAT
2881  CGCAGGTGTGGATATAGACCCACCCGCAGAGGAT
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Fig. 2. Nucleotide and deduced amino acid sequence of the *bgl*B gene from *T. maritima* MSB8 with its flanking region. The putative Shine-Dalgarno (SD) sequence and the conserved region of the glycosyl hydrolase family 3 are underlined. Arrowheads (E232A and D242A) indicate the residues exchanged by site-directed mutagenesis.

Table 1. Pairwise similarity between BglB and other β-glucosidases by amino acid sequence.

β-glucosidases[b]	Similarity (%) with β-glucosidase amino acid sequences[a]									
	1	2	3	4	5	6	7	8	9	10
BglB	100.0	39.5	35.5	42.5	86.1	47.0	42.7	39.1	37.6	44.0
AAC05445		100.0	36.8	37.2	40.0	33.5	54.3	40.0	44.3	55.7
AAO78420			100.0	35.1	34.8	31.7	36.9	35.1	34.2	38.5
AF0341				100.0	41.9	39.3	38.9	37.9	34.8	39.6
CAB01407					100.0	46.7	43.1	37.4	37.3	44.6
CAC07184						100.0	34.2	35.2	35.3	35.5
P14002							100.0	40.1	44.7	97.7
P27034								100.0	40.8	41.9
YP_119169									100.0	46.0
ZP_00503602										100.0

[a]Calculated with CLUSTAL W and the PAM250 residue weight table.

[b]The sequences are from the following sources: BglB, *Thermotoga maritima* MSB8 β-glucosidase; AAC05445, *Ruminococcus albus* β-glucosidase; AAO78420, *Bacteroides thetaiotaomicron* YPI-5482 thermostable β-glucosidase; AF0341, *Yersinia pestis* C092 putative β-glucosidase; CAB01407, *Thermotoga neapolitana* β-glucosidase; CAC07184, *Prevotella albensis* exo-1,4-β-glucosidase; P14002, *Clostridium thermocellum* thermostable cellobiase; P27034, *Agrobacterium tumefaciens* cellobiase; YP_119169, *Norcadia farcinica* IFM 10152 β-glucosidase; ZP_00503602, *Clostridium thermocellum* ATCC 27405 glycoside hydrolase family 3.

the purification steps were analyzed by MUG-non-denaturing-PAGE and SDS-PAGE, and only one protein band (approximately 81 kDa) was present after the final purification step (Fig. 3). The effect of pH on the activity of BglB against pNPG was determined at 80°C in various buffers ranging from pH 2.0 to 10.0 (Fig. 4A). Maximal activity was observed at pH 7.0. The temperature dependence of BglB activity toward pNPG was determined by measuring activity at various temperatures at pH 7.0. Maximal activity was observed at 80°C (Fig. 4B). Thermostability data were obtained by pre-incubating BglB at various temperatures and then measuring residual pNPG hydrolyzing activity under the standard assay condition. The measurement of BglB activity on pNPG showed that, even after 5 hrs of preincubation at 70, 80, and 90°C in 50 mM sodium phosphate buffer (pH 7.0), the enzyme retained about 97% of the initial activity (Fig. 4C). The effect of divalent cations such as Hg^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} were measured on pNPG at 80°C and pH 7.0. Hg^{2+}, Zn^{2+}, and Cu^{2+} inhibited enzyme activity on concentration of 50 mM (Fig. 4D). Substrate specificity of BglB was assayed for 30 min at 80°C against pNPG, salicin, arbutin, and cellobiose. However, BglB hydrolyzed substrates excepta cellobiose (Table 2).

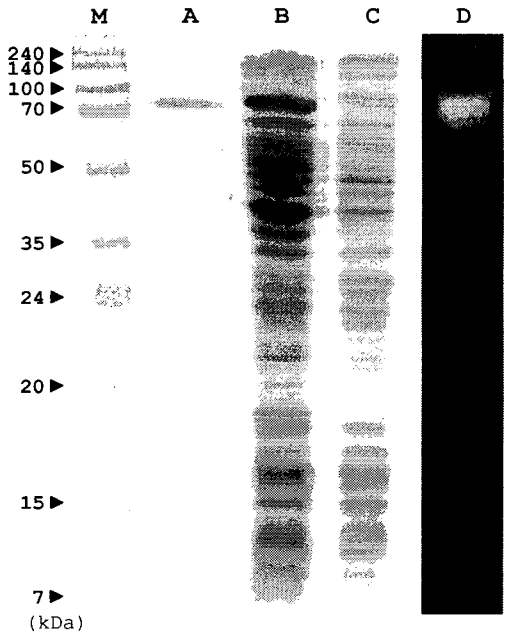

Fig. 3. Detection of BglB by SDS-PAGE. The purified protein was confirmed by SDS-PAGE. The gel was stained with Coomassie blue R-250. Lane a: Molecular weight markers. Lane b: purified BglB. Lane c: crude extract from IPTG induced BL21 containing pET-21a(+)/BglB. Lane d: crude extract from BL21 containing pET-21a(+)/BglB. Lane e: active band was detected using MUG as substrate.

Identification of essential residues for β-glucosidase activity

In vitro site-directed mutagenesis can be invaluable for studying protein structure-function relationships. Following site-directed mutagenesis and transformation into *E. coli*, possible mutant colonies were isolated and sequenced to identify the presence of the desired mutation. In this present study, we used site-directed mutagenesis to replace both Glu and Asp residues with Ala at sites in BglB that

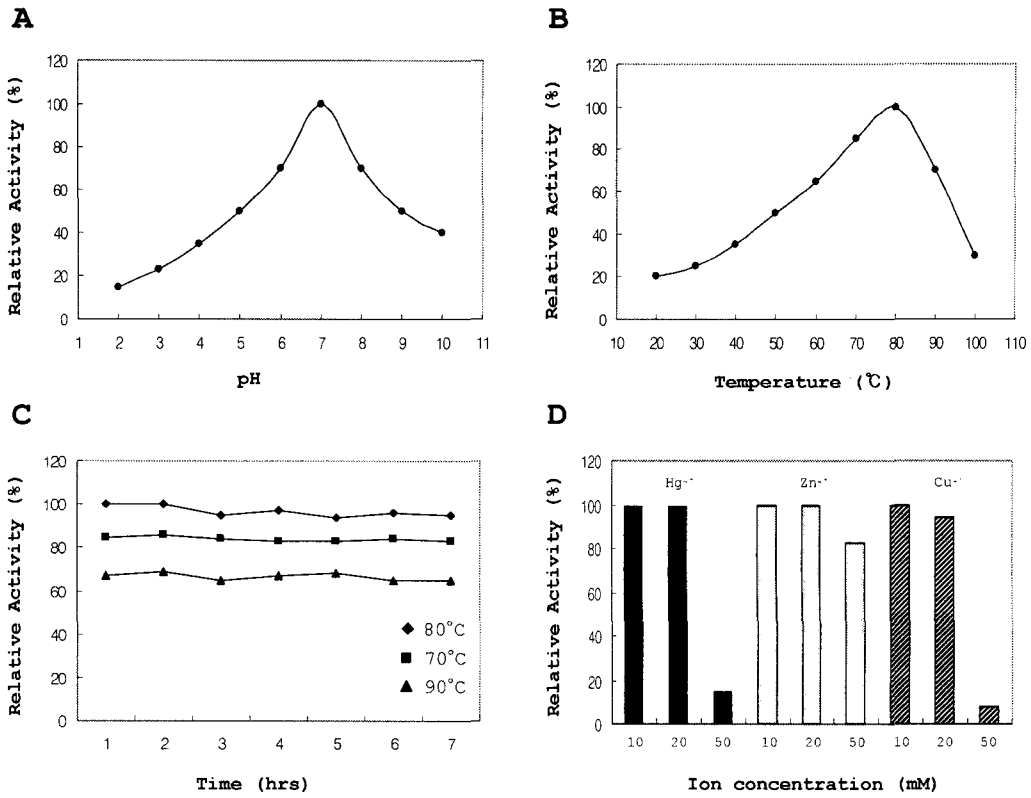

Fig. 4. Effect of pH, temperature on the activity of BglB. (A) Effect of pH on the relative activity of BglB. Enzyme activity was assayed at 80°C for 30 min in sodium phosphate buffers of indicated pH. (B) Effect of temperature on the relative activity of BglB. Enzyme activity was assayed at pH 7.0 for 30 min at the indicated temperature. (C) Effect of time and temperature on the relative activity of BglB. Enzymatic activity was assayed at 70 (■), 80 (◆), and 90°C (▲) at pH 7.0 for 5 hrs at the indicated reaction times. (D) Effect of concentration of metal ions Hg^{2+}, Zn^{2+}, and Cu^{2+} on the relative activity of BglB.

Table 2. Substrate specificity of BglB.

Substrates	Activity[a]
p-Nitrophenyl β-D-glucopyranoside (pNPG)	100
4-Hydroxyphenyl β-D-glucopyranoside (arbutin)	72
2-(Hydroxymethyl) phenyl β-D-glucopyranoside (salicin)	87
Cellobiose	0

[a]Micromoles of substrates hydrolyzed min^{-1} mg^{-1} (protein).

might serve catalytic function. Mutant plasmids were sequenced on both strands to confirm that only the intended mutation was introduced. Enzymatic activity assays were then performed with BglB mutants carrying E232A and D242A. These mutations abolished enzymatic activity for the β-glucoside (Fig. 1). These results indicate that Glu-232 and Asp-242, which are both conserved in the β-glucosidase sequences of family 3, are essential for BglB activity.

Discussion

The study of hyperthermophiles is engaging because of the hyperthermophilicity of the organisms and the hyperthermostability of their enzymes[25,37]. Hyperthermophilic enzymes can serve as model systems for understanding molecular mechanisms for protein stability, folding, evolution, and the upper temperature limit for enzyme function, thermodynamics in general[40]. *T. maritima* belongs to hyperthermophilic heterotroph, anaerobe by 16S rRNA gene analysis and phylogenetic tree[38]. Seven percent of their whole genome encodes glycosyl hydrolases involved in the metabolism of a monosaccharides or polysaccharides[27,43]. Thus genes can use sugars can be expected to respond to carbon source availability[27].

It was found that the open reading frame of *bglB* gene for the β-glucosidase from *T. maritima* contains 2,166 nucleotide sequences encoding a protein of 721 amino acids. BglA has a polypeptide of 446 amino acids coded by 1,341 bp with a predicted molecular mass of 51,545 Da[23].

Examination of amino acid sequences of BglB and BglA led to the prediction that it would be intracellular enzymes. It does not have a typical prokaryotic signal peptide.

The enzymatic activity of *bgl*B gene product was not shown by the method of MUG-SDS-PAGE but the *bgl*B-encoded enzyme exhibited a single band by the method of MUG-native-PAGE (Fig. 3). Thus, it seems that the protein may not be renatured or refolded after electrophoresis. After native-PAGE, the gel was immersed in the buffer containing MUG, and eluted a fluorescent band by the method of Lim *et al.* (data not shown)[25]. The concentrated protein was confirmed by SDS-PAGE (Fig. 3). An approximate molecular mass of BglB was 81 kDa, which corresponds well with the calculated molecular mass of 80,964 Da. The BglB is similar in size to other bacterial β-glucosidase such as 85 kDa enzyme of *Clostridium stercorarium*, have been reported[6]. The molecular weight of BglA was determined by Gabelsberger *et al.*[13,14] The size calculated from the amino acid sequence agrees reasonably well with the size (47 kDa) determined by SDS-PAGE.

The amino acid sequence of BglB has less similarity to other β-glucosidases below 50% homology except β-glucosidase (BglB) of *Thermotoga neapolitana* belongs to glycosyl hydrolase family 3. Pairwise similarity analysis revealed that BglB score below than 50% was found between that of *Prevotella albensis* (46.6%), of *Yersinia pestis* (43.6%), and of *Clostridium thermocellum* (44.5%). Comparison of the amino acid sequences of the BglA revealed that this enzyme is a member of the glycosyl hydrolase family 1. Identity scores higher than 50% over the entire length of the aligned enzyme pairs were found between of *Clostridium thermocellum* (52.7%), of *Thermoanaerobacter ethanolicus* ATCC 33223 (54.3%), and of *Bacillus circulans* (50.2%).

BglA has broad substrate specificity, which is active against various β-glucosides and β-galactosides[13]. Substrate specificity of BglB also examined with a variety of β-linked saccharide derivatives. Enzyme activity was assayed against nitrophenyl glycosides and other glucosides such as a cellobiose, salicin, and arbutin for 30 min at 80°C. BglA and BglB hydrolyzed most β-linked glucosides, whereas, BglB was not able to hydrolyze cellobiose. The optimal activity of BglB observed at pH 7.0 and 80°C. Otherwise, Goyal *et al.*[15] determined using a Na-citrate buffer and Na-succinate buffer at 30 or 85°C for enzyme activity. Enzymatic activity was exhibited optimum pH 3.6 at 30°C and pH 5.0-5.2 at 85°C, respectively, which was showed the distinct characteristics. Also, *Agrobacterium tumefaciens-Thermotoga maritima* chimeric enzyme activity was observed on around pH 6.2-6.5 and 45°C[16].

BglB is clearly belongs to glycosyl hydrolase family 3, which catalyst the reaction of enzymes by a displacement mechanism[12,18]. It was confirmed that the Asp and Glu residues of BglB was essential for the β-glucosidase activity of *T. maritima* using the site-directed mutagenesis method. The glutamic acid and aspartic acid are an important amino acid residues within a conserved region characterized by the consensus sequence [LIVM](2)-[KR]-x-[EQK]-x(4)-G-[LIVMFT]-[LIVT]-[LIVMF]-[ST]-D-x(2)-[SGADNI] (X is an any residue) from amino acid sequences of glycosyl hydrolase family 3 (website; http://kr.expasy.org/cgi-bin/nicedoc.pl?PDOC00621). As a result, aspartic acid (D) and glutamic acid (E) residue in the conserved VLREEWGFDGFVMSDWYA region was predicted to be directly involved in the hydrolysis of β-glucosidic bonds from *T. maritima* strain MSB8 by acting as a nucleophile and acid/base. Based on the multi-alignment of amino acid residues of glycosyl hydrolase family 3 enzymes of *Hordeum vulgare* (accession number 1EX1_A)[41] (data not shown), it is found that an 'SDW' sequence is highly conserved (Fig. 5)[22]. However, amino acid sequence of BglB identified the low similarity (16%) with β-glucosidase from *Hordeum vulgare*. Based on 3D structure of *Hordeum vulgare* β-glucosidase, computer modeling by the Cn3D 4.0 version program predicted that BglB is an (α_{12}/β_{10}). The active center of BglB is located in the cavity formed by polypeptide loops. Therefore, we suggest that one conserved aspartic acid residue, Asp-242, participate in BglB catalysis, acting as a proton donor and a nucleophile (Fig. 5). The Asp residue has been identified as the nucleophile in the *Aspergillus niger*[10] and *A. wentii*[21] β-glucosidases by active-site affinity labeling[22]. Point mutation of Glu-232, is conserved in motif sequence VLREEWGFDGFVMSDWYA, even though there was no activity exhibited, although Glu-232 on the simulated 3D structure was located on outside the loop. Two residues might play an important role as an acid/base and nucleophile in catalysis. Thus, further study is required to determine the amino acid residues involve in catalytic activity by simulation of the 3D structure of BglB.

Acknowledgment

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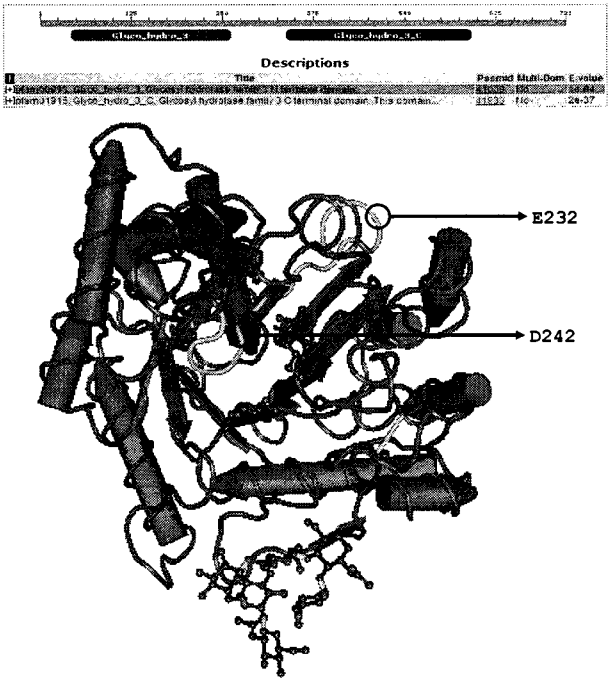

Fig. 5. Modular domain and 3D structure predicted for BglB. The modular domain predicted by RPS-Blast (NCBI). The numbers indicate the protein residues. Two bars indicate the relative position and the size of the glycosyl hydrolase family 3 N-terminal domain and C-terminal domain. 3-dimensional structure of BglB predicted and viewed by Cn3D 4.0 version program (NCBI).

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초록 : *Thermotoga maritima*로부터 고온성 β-glucosidase (BglB)의 클로닝과 필수아미노산 잔기의 확인

홍수영 · 조계만 · 김용희 · 홍선주 · 조수정[2] · 조용운[2] · 김훈[3] · 윤한대[1*]

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초고온성 세균인 *Thermotoga maritima*로부터 β-glucosidase 유전자를 클로닝한 후 대장균 숙주에서 발현시켰다. 이 효소는 salicin, arbutin, pNPG과 같은 탄소원의 β-글루코시드 결합을 가수분해하였다. 721개의 아미노산을 암호화하는 2,166 bp의 DNA 염기서열로된 유전자이였다. 다른 β-glucosidase 효소들과 단백질 유사성을 비교한 결과 glycosyl hydrolase family 3에 속하였으며 MUG-nondenaturing PAGE와 SDS-PAGE에 의해 확인된 단백질의 크기는 약 81 kDa이었다. 효소활성은 pH 7.0, 80℃에서 가장 높은 활성을 나타냈으며 이 효소의 아미노산 서열에 있는 두 개의 아미노산 잔기 (232번 글루탐산과 242번 아스파르트산 잔기)를 알라닌으로 치환시켜 활성이 없어지는 것으로 보아 이 두 잔기가 효소활성에 중요한 역할을 하는 것으로 추정된다.