Metabolism of Ginsenoside Rb1 by Human Intestinal Microflora and Cloning of Its Metabolizing β-D-Glucosidase from *Bifidobacterium longum* H-1

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To understand the role of intestinal microflora in expressing the pharmacological effect of ginsenoside Rb1, the metabolic activity of ginsenoside Rb1 by 148 fecal specimens was measured and its metabolizing β-glucosidase was cloned. The average activities for *p*-nitrophenyl-β-D-glucopyranoside and ginsenoside Rb1 were 0.097±0.059 μmol/min/mg and 0.311±0.118 pmol/min/mg, respectively. These enzyme activities were not different between male and female, or between ages. A gene encoding β-D-glucosidase (BglX) was cloned from *Bifidobacterium longum* H-1, which transformed ginsenoside Rb1 to compound K. The probe for cloning was synthesized from the genes encoding a β-D-glucosidase of previously reported *B. longum* DJO10A. The sequences of the cloned gene revealed 2364 bp open reading frame (ORF) encoding a protein containing 787 amino acids (molecular weight of 95 kDa). The gene exhibited 99% homology (identities) to that of *B. longum*. The cloned gene was expressed under T7 promoter of the expression vector, pET-39b(+), in *Escherichia coli* BL21(DE3), and the expressed enzyme was purified by using HiTrap immobilized metal affinity chromatography (IMAC) HP. The enzyme potently biotransformed ginsenoside Rb1, loganin, arctiin and arbutin to ginsenoside Rd, loganetin, arctigenin and hydroquinone, respectively, but was not active in the case of hesperidin, and kakkalide. This is the first report on cloning and expression of β-D-glucosidase from *B. longum*. Based on these findings, ginsenoside Rb1 may be metabolized to bioactive compound(s) by exo-β-D-glucosidase(s) produced from the intestinal bacteria and its pharmacological effects may be dependent on intestinal bacterial exo-β-D-glucosidase(s) activity.

Key words intestinal microflora; metabolism; ginsenoside Rb1; *Bifidobacterium longum* H-1; β-D-glucosidase; β-D-glucosidase-coding gene

Most of the herbs are orally administered to humans. The components of the oral herbal medicines are therefore inevitably brought into contact with intestinal microflora in the alimentary tract. The intestinal bacteria may transform these components before they get absorbed from the gastrointestinal tract. Studies on the metabolism of the herbal components by human intestinal microflora are of a great importance in terms of gaining an understanding on their biological effects.[1,2] In relation to the role of intestinal microflora on the pharmacological actions of herbal medicines, Kobashi *et al.* reported that some drug-metabolic enzyme activities of intestinal bacteria were significantly different between Jitsu-syo and Kyo-syo Japanese, although the intestinal bacteria between Jitsu-syo and Kyo-syo Japaneses were not different.[3] We have reported that some fecal bacterial enzymatic activities related to the pharmacological actions of herbal medicines including ginseng were variable among individuals.[4–7] Nevertheless, studies on the metabolic activities of herbal medicine components by human intestinal microflora are not sufficient.

Ginseng (the root of *Panax ginseng* C.A. Meyer, Araliaceae), which contains ginsenosides as major constituents, is frequently used as a traditional medicine and in Asian countries.[8,9] The ginsenosides have been reported to show various biological activities including anti-inflammatory activity[10] and anti-tumor effects.[11] To express the afore mentioned pharmacological actions, it is presumed that ginseng saponins must be metabolized by human intestinal bacteria after being taken orally.[12,13] Thus, ginsenosides Rb1, Rb2 and Rc are transformed to 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (compound K) by human intestinal bacteria and absorbed into the blood.[14–18] The transformed compound K exhibits the potent antitumor, anti-inflammatory, and antiallergic actions more than ginsenoside Rb1.[12,19–21] Therefore, intestinal bacteria may play the important role in expressing the pharmacological effects of herbal medicines, such as ginseng.

Based on the biotransformation of ginsenoside Rb1, *Bifidobacterium* species from human intestinal bacteria were isolated,[16] Amongst them, *B. logum* H-1 potently transformed ginsenosides to compound K in a most potent manner. However, the general and genetic properties of H-1 gene encoding β-D-glucosidase(s) (BglX(s)) catalyzing the metabolic reactions have also not been studied.

Therefore, to understand its pharmacological effects, we determined the metabolic activity of ginsenoside Rb1, a main constituent of ginseng, by human fecal suspension, and investigated its difference between male and female, or between ages. Furthermore, we cloned the gene encoding a β-D-glucosidase from *B. longum* H-1, a ginsenoside Rb1-metabolizing anaerobic microbe from human feces, and in particular characterized its substrate specificity in terms of hydrolyzing natural glycosides.

MATERIALS AND METHODS

Materials Hesperidin, baicalin, arbutin and all antibiotics used in this study were obtained from Sigma Aldrich, Inc. (St. Louis, MO, U.S.A.). Isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), DNA ladder marker, protein molecular weight marker, and all restriction enzymes used in this study were obtained from Fermentas Co. (Burlington, ON, Canada). Wizard Genomic DNA Purification Kit and pGEM-T easy

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vector system were purchased from Promega Co. (Madison, WI, U.S.A.). *ExTaq*DNA polymerase and T4 ligase system were obtained from Takara Co. (Tokyo, Japan). Gel Extraction Kit and Plasmid DNA Purification Kit were from Qiagen GmbH (Hilden, Germany). Immobilized metal affinity chromatography (IMAC) column was purchased from GE Healthcare (Uppsala, Sweden). Loganin, were purchased from Extrasynthese (Cedex, France). Arctiin, mangeiferin and kakkalide were isolated according to the previous method of Lee *et al.*,[22] Jung *et al.*[23] and Han *et al.*,[24] respectively. Ginsenoside Rb1 (purity, >95%) and compound K (purity, 95%) were isolated using the previously published method of Bae *et al.*[20]

Bacterial Strains and Vectors *B. longum* H-1 was isolated from human feces (Korean Culture Collection No.KCCM-10493, Seoul, Korea). *B. longum* H-1 was cultured in GAM broth (Nissui Pharmaceutical Co., Tokyo, Japan) and *Escherichia coli* strains BL21 (DE3), and JM109 (RBC bioscience Co., Bangkok, Thailand) were employed for gene cloning and expression, respectively. Luria–Bertani broth (LB) was used as the growth medium for all the *E. coli* strains. The plasmid vectors used in this study were pGEM-T easy (Promega Co., Madison, WI, U.S.A.), and pET 39b(+) (Novagen, San Diego, CA, U.S.A.). The recombinant enterokinase was obtained from Novagen.

Subjects The subjects were 148 healthy Korean persons [average 49.89±16.92 years; range, 21–77 years; 87 males (51.66±16.86 years: 18 twenties, 17 thirties, 9 forties, 13 fifties, 21 sixties and 9 seventies) and 61 females (48.66±16.941 years: 11 twenties, 6 thirties, 8 forties, 8 fifties, 19 sixties and 9 seventies)]. Exclusion criteria included smoking and current medication, especially regular or current use of antibiotics. The recruit of subjects and collection of their stools were approved by the Committee for the Care and Use of Clinical Study in the Medical School, Kyung Hee University.

Specimen Preparation The human fecal specimens (about 1 g) prepared according to a previous method,[6] were collected in plastic cups 9 h after fasting, and then carefully mixed with a spatula and suspended with cold 9 mL saline. Fecal bacterial suspension was centrifuged at 500×***g*** for 5 min. The resulting supernatant was sonicated for 10 min and then centrifuged at 10000×***g*** for 20 min. The resulting supernatant was used for the assay of enzyme activity. The fecal suspension was centrifuged at 500×***g*** for 5 min. The supernatant was then centrifuged at 10000×***g*** for 20 min. The resulting precipitates were used as a metabolic enzyme source for the assay of enzyme activity. The preparation and assay of the enzyme source were performed within 24 h.

Molecular Cloning of the Genes Encoding a *β*-D-Glucosidase from *B. longum* H-1 The genomic DNA of *Bifidobacterium longum* H-1 was isolated by using the Wizard genomic DNA purification Kit (Promega). The core region of the *β*-D-glucosidase was obtained through polymerase chain reaction (PCR) by employing degenerate primers, which were designed on the basis of two conserved amino acid sequences amongst *β*-D-glucosidase from *B. longum* DJO10A (GenBank protein accession number NC_010816), *B. longum* JDM301 (GenBank protein accession number NC_014169) and *B. longum* BBMN68 (GenBank protein accession number NC_014656). Sequences of internal amino acids and primers synthesized from the conserved amino acid sequences are listed in Table 1. The PCR product was labeled with digoxigenin (DIG) and used as a hybridization probe. In order to prepare the genomic library of *B. longum* H-1, the chromosomal DNA was partially digested with *Bam*HI at the 5′ end and with *Hin*dIII at the 5′ end. A portion of the digested chromosomal fragments (1.5- to 4-kb) was removed from the gel. The DNA was extracted and ligated into the pKF3 vector, which was subsequently digested with the same enzymes and transformed into competent *E. coli* TH2 cells. The positive clones, as screened by Southern blotting, were sequenced, and the complete full-length gene encoding *β*-D-glucosidase was analyzed by using Blast (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) and open reading frame (ORF) finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Expression of *β*-D-Glucosidase Genes in *E. coli* The entire open reading frames of BglX were amplified from the original clone in pET-39b(+) vector (Novagen). The constructs were transformed into *E. coli* JM109 competent cells (Promega), and following confirmation of the correct sequence, the recombinant plasmids were retransformed into *E. coli* BL21 (DE3). For the facile expression of BglX, the pET 39b(+) expression system contained an inducible T7 promoter. The *β*-D-glucosidase expressed in BglX -clone *E. coli* was purified by culturing the bacteria to the mid-log phase (OD_{600} 0.6–1) in 300 mL of LB broth containing 30 mg/mL of kanamycin. The cells were then induced with 0.5 mM IPTG at 20°C for 20 h. The cultured cells were centrifuged at 6000×***g*** for 30 min at 4°C and the supernatant was discarded. The cell

Table 1. Oligopeptide and Synthesized Primer Used in This Study

Name	Sequence
Oligopeptide sequences	
BglX F-1	KHFA
BglX R-1	WLLTD
Primers synthesized from the oligopeptides	
Primer BglX F-1	AARCASTTYGCN
Primer BglX R-1	RTCNGTNARNARCCA
Primers used for cloning	
Primer BglX F-2	GAG AGA GGA TCC NAT GAC CGA CAA CAC TA
Primer BglX R-2	GAG AGA AAG CTT CTA CGC CAC GGT GAA

Mix-base codes for degenerate primers: N, A/G/C/T; R, A/G;Y, C/T; M, A/C; S, G/C restriction enzyme sites for cloning are underlined: GGATCC, *Bam*HI site; AAGCTT, *Hin*dIII site.

Table 2. Comparison of Nucletide and Amino Acid Sequence of *β*-D-Glucosidase from *Bifidobacterium* sp.

Microbe	Homology[a] (%)	
	Nucleotide	Amino acid
B. longum NCC2705	99	99
B. longum JDM301	97	99
B. longum DJO10A	95	98
B. dentium Bd1	81	83
B. adolescentis ATCC15703	79	83
B. longum subsp. *longum* KACC91563	42	28

a) The homology was determined by BLAST.

pellet was resuspended in 20 mL of 1× binding buffer (20 mM sodium phosphate buffer containing 300 mM NaCl and pH 7.2) and was sonicated in a tube on salt-ice bath. After sonication, centrifugation at 14000×***g*** for 30 min was carried out to remove debris.

Purification of Expressed BglX Purification of *β*-D-glucosidase under native conditions was performed by using HiTrap IMAC HP. The 20 mL of recombinant BglX (rBglX) was loaded onto the HiTrap IMAC HP, charged with 4 column volumes (CV) of 1× charge buffer (0.1 M $NiSO_4$). The rBglX was eluted by using a combination of stepwise and linear gradients of 0 to 500 mM imidazole in 20 mM sodium phosphate buffer with 300 mM NaCl (pH 7.2). Each individual active fraction was dialyzed in 20 mM sodium phosphate buffer (pH 7.2). The dialyzed solutions were incubated with recombinant enterokinase at 20°C for 12 h. The cleaved protein mixture was then loaded onto the His Bind Column and eluted with 1× binding buffer.

Protein Determination and Electrophoresis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed that the rBglX in the eluted fractions were homogenous proteins in which the fusion segment (DsbA) was completely removed. The Bradford assay was used to calculate protein concentration by employing bovine serum albumin as a standard.[25] SDS-PAGE was performed by using a discontinuous polyacrylamide gel (10% separating gel and 3% stacking gel with 1-mm thickness) under denaturing conditions. The gel was stained with Coomassie brilliant blue R250. The molecular weights of the purified enzymes were estimated by comparison with standard molecular weight markers.

Characterization of Recombinant Enzymes In order to establish the standard reaction conditions for the enzymes, several enzymatic properties were analyzed by using *p*-nitrophenyl-*β*-L-glucopyranoside (pNGp) as substrate. The optimum pH for the enzyme activity was determined by incubation at 37°C for 15 min in the pH range from 4.0 to 8.5 with 0.5 pH unit interval. The following buffers were used: 20 mM sodium acetate, pH 4.0 to 5.5; 20 mM sodium phosphate, pH 5.5 to 7.5; and 20 mM Tris–HCl, pH 7.5 to 8.5. The effect of temperature on enzymatic activity was determined by carrying out the reaction at various temperatures ranging from 20 to 70°C in the presence of optimum pH buffer. The results were expressed as percentages of the activity obtained at either the optimum pH or the optimum temperature. The effect of NaCl on the activity of the purified rBglX was examined. Enzymes used for the determination of the effect of ionic strength were desalted by dialysis and concentrated before use. The enzyme activities at 37°C were measured in the presence of various concentrations of NaCl in 20 mM sodium phosphate buffer (pH 7.2). To prevent precipitation, 20 mM sodium phosphate (pH 7.0) containing optimal NaCl was used as the buffer in these assays, and the enzyme was also dissolved in the same buffer. The effects of divalent metal cations ($BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$ and $NiCl_2$ each at 1 mM) on the activity of the purified enzyme were also determined. To prevent precipitation, 50 mM Tris–HCl (pH 7.0) containing optimal concentration of NaCl was used as the buffer in these assays, and the enzyme was also dissolved in the same buffer. The relative activities were expressed as a percent of the activity *versus* the control reaction.

Assay of Specific Activities and Kinetic Parameters In the case of rBglX, a reaction mixture containing 350 μL of 50 mM sodium acetate buffer, pH 5.5, 100 μL of 1 mM *p*-nitrophenyl-*β*-D-glucopyranoside and 50 μL of rBglX was incubated for 10 min at 37°C in the shaking water bath. The reaction was stopped by adding 500 μL of 0.5 N NaOH. The absorbance of each reaction mixture was measured at 405 nm and enzyme activity was calculated. Specific activity was defined in terms of units/mg of protein. Kinetic parameters (K_m and V_{max}) were determined by using variable substrate concentrations (0.25, 0.5, 0.75, 1, 1.25, 1.5 mM) under standard reaction conditions. Kinetic parameters were derived from Michaelis–Menten representations.

Assay of Natural Glycosides-Hydrolyzing Activity A reaction mixture, containing 175 μL of 50 mM phosphate buffer, pH 7.2, 50 μL of 1 mM natural glycosides (ginsenoside Rb1, loganin, arctiin, mangiferin, kakkalide, hesperidin, baicalin or arbutin) and 25 μL of the recombinant enzymes, was incubated for 30 min, 1 and 6 h at 37°C, respectively. The reaction was stopped by adding 250 μL of MeOH, the reaction mixture was cooled and the parental compounds and its metabolites were analyzed by Hewlett Packard Series 1050 HPLC system. The analytical column was an Agilent Hypersil ODS (100×4.6 mm i.d., 5 μm; Agilent Technologies, U.S.A.) protected by a C18 Security Guard Cartridge (Phenomenex, Torrance, CA, U.S.A.). The elution solvent was acetonitrile (ACN) and double distilled water (DDW). Separations were performed with a linear gradient of 0–70% ACN in DDW for 15 min at a flow rate of 1.0 mL/min and the detection of ginsenosides Rb1 and Rd was carried out at 203 nm; a linear gradient of 30–70% MeOH in DDW for 7.5 min at a flow rate of 1.0 mL/min and arctiin and arctigenin were detected at 254 nm; and with a linear gradient of 10–26% ACN in DDW for 21 min at a flow rate of 1.0 mL/min the detection of loganin and loganetin at 236 nm was achieved. A sample volume of 20 μL was used for injection. The retention times of ginsenoside Rb1, ginsenoside Rd, arctiin, arctigenin, loganin, and loganetin were 10.0, 11.4, 5.2, 6.7, 5.1, and 11.8 min, respectively. Calibration curves were preformed in the range of 0.005–0.05 mM for ginsenoside Rb1 and ginsenoside Rd, 0.002–0.02 mM for arctiin, arctigenin, loganin, and hydroquinone, and 0.01–0.1 mM for arbutin and loganetin.

Statistics The SPSSwin 8.0 program was used for statistical analysis of the data. The differences in fecal enzyme activities between males and females and between ages were assessed by analysis of variance (ANOVA).

RESULTS

Metabolic Activities of Ginsenoside Rb1 by Human fecal Suspensions To understand the role of human intestinal microflora in expressing the pharmacological effect of ginseng constituents, we measured ginsenoside Rb1-metabolic activity (Fig. 1). When the *β*-glucosidase activities of 148 human fecal suspensions for *p*-nitrophenyl-*β*-D-glucopyranoside was measured, the *β*-glucosidase activity in the tested specimens was 0–0.278 μmol/min/mg. The average *β*-glucosidase activities (mean±S.D.) of total, female and male specimens were 0.097±0.059, 0.100±0.057 and 0.094±0.061 μmol/min/mg, respectively. The *β*-glucosidase activity was not different between males and females, or between ages. When the metabolic activities of these fecal specimens for ginsenoside Rb1

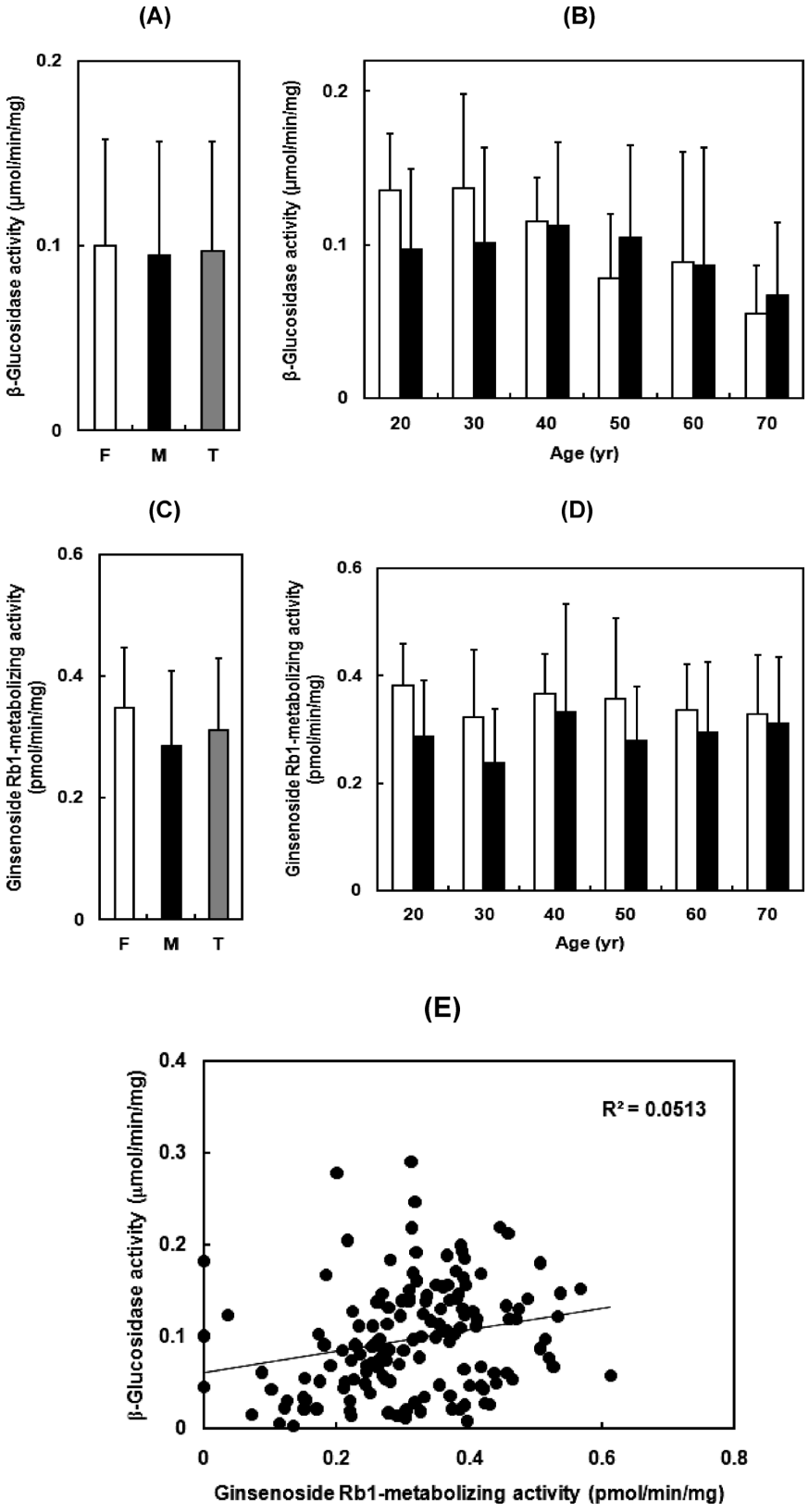

Fig. 1. Fecal β-Glucosidase and Ginsenoside Rb1-Metabolic Activities of 148 Individuals

(A) Average β-glucosidase activity (white bar, female; black bar, male; gray bar, total specimens). (B) Distribution of β-glucosidase activity by sex and ages. (C) Average ginsenoside Rb1-metabolic activity (white bar, female; black bar, male; gray bar, total specimens). (D) Distribution of ginsenoside Rb1-metabolic activity by sex and ages. (E) Profile of the relationship between ginsenoside Rb1-metabolic activity and β-glucosidase activity.

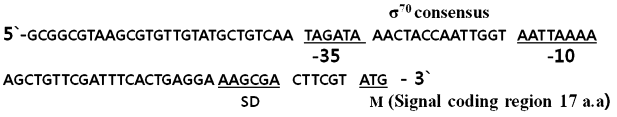

Fig. 2. Nucleotide Sequence of the *B. longum* rBglX Gene Upstream Region

The proposed Shine–Dalgarno (SD) sequence and the consensus sequence for the sigma 70-type promoter are underlined.

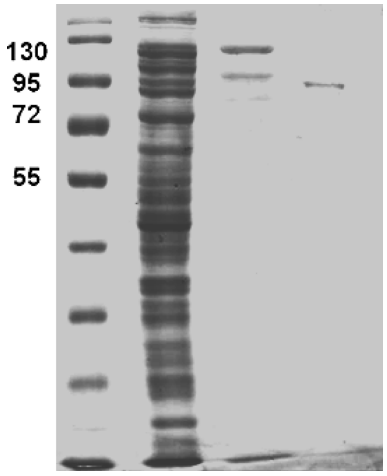

Fig. 3. SDS-PAGE of Purified rBglX

Lane 1, protein molecular mass marker; lane 2, soluble crude extract; lane 3, rBglX after purification by using IMAC chromatography; lane 4, rBglX after treatment with enterokinase and Ni-NTA His Bind Column.

was measured, the metabolic activity in the tested specimens were 0.01–0.612 pmol/min/mg. The average ginsenoside Rb1-metabolic activities of total, female and male specimens were 0.311±0.118, 0.348±0.099, and 0.285±0.124 pmol/min/mg, respectively. The ginsenoside Rb1-metabolic activity was also not different between males and females, or between ages. The ginsenoside Rb1-metabolic activities of tested specimens is in proportion to their β-glucosidase activities (R^2=0.0513).

Cloning and Sequencing of BglX from *B. longum* H-1 Next, to investigate the enzymatic properties of ginsenoside Rb1-metabolizing β-glucosidase of intestinal microflora, it was cloned from *B. longum* H-1, which metabolizes ginsenoside Rb1 to compound K. To clone it, two primers (a probe for cloning BglX gene) were designed based on the internal amino acid sequences of rBglX of *B. longum* DJO10A and amino acid sequences from multiple alignments of other species. By using the PCR product (2.4 kb) prepared by these primers, the BglX gene was identified in an approximately 3-kb *Bam*HI/*Hin*dIII fragment of *B. longum* H-1 genomic DNA by Southern blot analysis. A gene library containing 2- to 4-kb *Bam*HI/*Hin*dIII fragments was constructed and screened with the same probe. One positive clone containing an approximately 3-kb *Bam*HI/*Hin*dIII fragment was isolated from the gene library. Sequencing of the clone showed that it contained the full BglX gene and a partial sequence of the nucleotidyltransferase gene, as predicted by BLAST search and ORF finder. The nucleotide sequence of the BglX, which is the gene encoding β-D-glucosidase of *B. longum* H-1, consisting of a 2364 bp sequence encoding a protein with 787 amino acid residues containing a signal peptide (17 amino acids) was determined by using SignalP ver. 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The putative ribosome-binding site (Shine–Dalgarno sequence, AAGCGA) was identified to be located 7 bp upstream from the start codon ATG by using Glimmer ver 2.02 (http://nbc11.biologie.uni-kl.de/framed/left/menu/auto/right/glimmer2.02/). The putative sigma 70 type promoter sequences of both TTTACA (resembling −35 sequence) and GGTTATTAT (resembling −10 sequence) were identified to be located at positions −75 and −55, respectively by using BPROM (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) (GenBank accession No. HQ875470) (Fig. 2). It exhibited 95–99% homology to the β-glucosidases (BglX) of *B. longum* NCC2705 (GenBank protein accession number AE014295), JDM 301 (GenBank protein accession number CP002010) and DJO10A (GenBank protein accession number NC010816), deposited in GenBank. The BglX gene sequence from *B. longum* H-1 showed 81 and 79% homologies to the gene sequences of β-glucosidase (Bgl2) from *B. dentium* Bd1 (GenBank protein accession number CP001750) and *B. adolescentis* ATCC15703 (GenBank protein accession number NC008618), deposited in GenBank, respectively. However, it did not exhibit homology (42%) to the gene sequence of β-glucosidase from *B. longum* subsp. *longum* KACC91563 (GenBank protein accession number CP002794).[26]

Expression and Purification of the rBglX In order to investigate the enzymatic property of the rBglX in *E. coli*, the

Table 3. Purification of Recombinant β-D-Glucosidase

Step	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Yield (%)	Fold
Crude extract	372.14	311.36	1.69	100	1
Ni-affinity column	51.87	3.75	13.85	14	8
Enterokinase treatment	18.52	0.84	21.96	5	13

Table 4. Effects of Divalent Metal Cations on the Activity of Recombinant β-D-Glucosidase

Metal	Relative activity (%)
None	100.0±2.7
Ba^{2+}	109.6±1.8
Ca^{2+}	102.3±4.2
Co^{2+}	95.4±5.8
Cu^{2+}	17.1±6.1
Fe^{2+}	93.4±7.6
Ni^{2+}	69.5±8.1
Mg^{2+}	96.4±4.2

The concentration of all divalent metal cations was 1 mM. The activity for *p*-nitrophenyl-β-D-glucopyranoside (21.96 μmol/min/mg) is considered as 100%. These data were calculated from triplicate experiments.

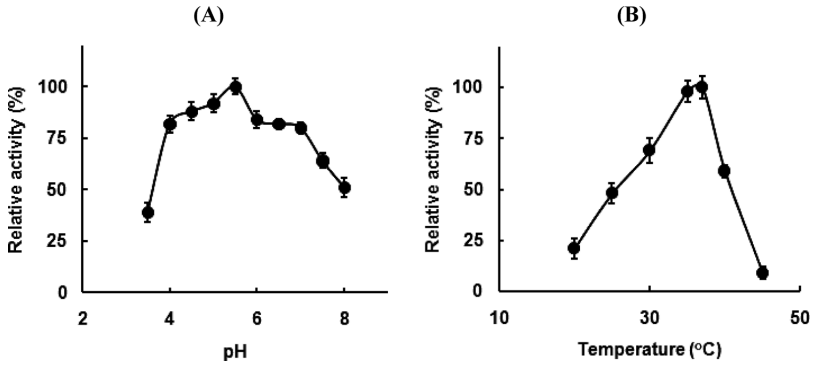

Fig. 4. Effects of pH (A) and Temperature (B) on rBglX Activity

These data were calculated for triplicate experiments.

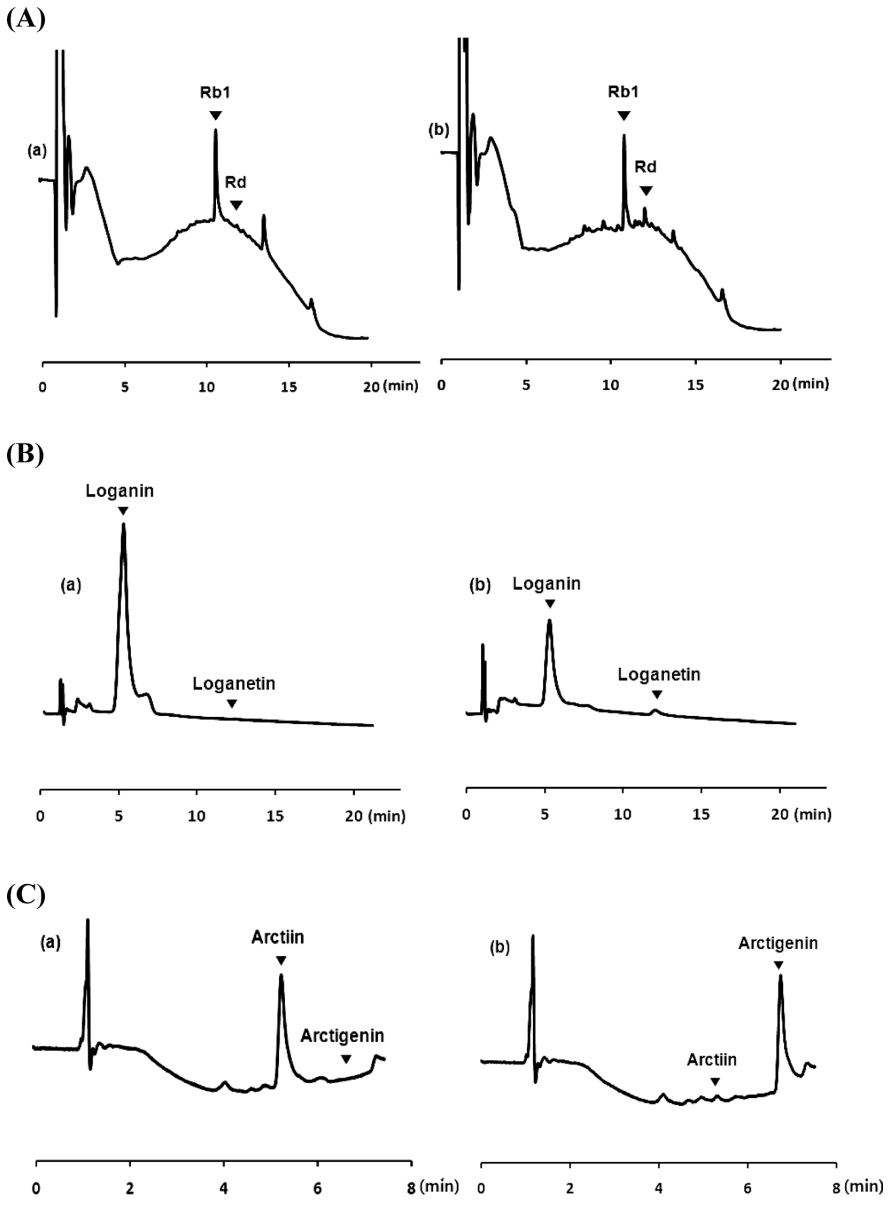

Fig. 5. Biotransformation of Ginsenoside Rb1, Loganin and Arctiin by rBglX

The reaction mixtures were incubated for 0.5 h (loganin and arctiin) or 4 h (ginsenoside Rb1) and the metabolites were analyzed before incubation (a) and after incubation (b) by HPLC. The details of reaction mixture are as follows: (A) ginsenoside Rb1 with rBglX; (B) loganin with rBglX; and (C) arctiin with rBglX.

rBglX was expressed by inserting the cloned gene along with the codon of its signal peptide into the *Kpn*I and *Hin*dIII sites of pET-39b(+) vector, which has N-terminal and C-terminal 6×His tag. The pET 39b(+) vector has DsbA, which is the periplasmic enzyme that catalyzes the formation and isomerization of disulfide bond and is used for the purpose of enhancing solubility. Purification of the recombinant wild-type and mutant proteins was facilitated by the presence of six histidine residues (6×His tag) at the N-terminus of the enzyme. This permitted us to use only a single step to purify the *β*-D-glucosidase by using Ni-HiTrap IMAC HP. The molecular weight of the purified *β*-D-glucosidase with the pET-39b(+) was determined to be 95 kDa by SDS-PAGE (Fig. 3). The fold purification of rBglX purified from the crude extract was 13-fold. The specific activity of the finally purified *β*-D-glucosidase was 21.96 *μ*mol/min/mg (Table 3).

General Property of rBglX When the activity of rBglX was assayed at 37°C by using PNG as a substrate, the optimal pH for rBglX activity was identified to be 5.5. When the enzyme was incubated for 15 min in optimum pH standard essay condition, the maximum temperature for rBglX activity was measured to be 35–37°C. There was a significant decrease in the activity of rBglX at temperatures below 20°C and above 50°C (Fig. 4). The activity of rBglX was not affected by the presence of NaCl (0 to 1 M). When the effects of various divalent metal cations (at 1 mM) on rBglX activity were investigated, most of the metals except for Cu^{2+} and Ni^{2+} had no effect on the enzymatic activity (Table 4). However, Cu^{2+} followed by Ni^{2+} potently inhibited rBglX activity.

Substrate Specificities of rBglX The substrate specificities of rBglX with respect to synthetic substrates and natural glycosides were investigated (Table 5). The best substrate for rBglX was identified to be *p*-nitrophenyl-*β*-D-glucopyranoside (pNPGp). The rBglX could not hydrolyzed the other glycosides containing sugar moieties, such as D-galacotose, D-glucuronic acid and L-rhamnose. To determine whether the rBglX could hydrolyze glucose-containing natural glycosides, we investigated the substrate specificities for loganin, arctiin, ginsenosides Rb1, hesperidin, mangiferin and kakkalide. It was observed that rBglX transformed loganin, arctiin, arbutin and ginsenoside Rb1 to loganetin, arctigenin, hydroquinone and ginsenoside Rd, respectively (Fig. 5). However, it could not biotranform mangiferin, hesperidin and kakkaldie. Amongst the afore mentioned substrates, arctiin was transformed to its alycone form, followed by loganin and ginsenoside Rb1.

Table 5. Substrate Specificity of Recombinant *β*-D-Glucosidase

Substrate	Activity (%)
p-Nitrophenyl-*β*-D-glucopyranoside	100
p-Nitrophenyl-*β*-D-galactopyranoside	0
p-Nitrophenyl-*β*-D-xylopyranoside	0
p-Nitrophenyl-*β*-D-celloside	0
p-Nitrophenyl-*β*-D-fucopyranoside	0
p-Nitrophenyl-*β*-D-arabinofuranoside	0
p-Nitrophenyl-*β*-D-rhammnopyranoside	0
p-Nitrophenyl-*α*-D-glucopyranoside	0
p-Nitrophenyl-*β*-D-arabinopyranoside	0
Ginsenoside Rb1	1.2
Loganin	11.0
Arctin	26.1
Arbutin	16.3
Baicalin	0
Mangiferin	0
Kakkalide	0
Hesperidin	0

The activity for *p*-nitrophenyl-*β*-D-glucopyranoside (21.96 *μ*mol/min/mg) is considered as 100%. These data were calculated from triplicate experiments.

Kinetic Constants of rBglX Michaelis–Menten constants were determined under optimum reaction conditions in experiments designed to calculate reaction velocities at each substrate concentration (Table 6). By using pNGp, ginsenoside Rb1, arctiin, and loganin as substrates for rBglX, the K_m and V_{max} were estimated to be 0.83 mM and 56.50 *μ*mol/min/mg, 4.33 mM and 4.08 *μ*mol/min/mg, 1.62 mM and 11.68 *μ*mol/min/mg, and 1.92 mM and 11.68 *μ*mol/min/mg, respectively.

DISCUSSION

All individuals possess their own characteristic indigenous strain of intestinal bacteria.[27,28] This is due to the affinity between the intestinal lumen of the individual and the bacteria. Newly ingested bacteria cannot necessarily colonize and proliferate in the intestine. These results are supported by previous reports that intestinal microflora in feces are thought to be rather stable over time within individuals in the absence of disease and antimicrobial therapy.[3,28,29] Orally administered hydrophilic constituents of herbal medicines are metabolized to bioactive compounds by intestinal microflora.[1,2] Therefore, intestinal microflora may play the important role in expressing the pharmacological activities of herbal medicines containing hydrophilic glycosides, such as ginsenoside Rb1. The ginsenoside Rb1 was metabolized to compound K by intestinal microflora and then the absorbed compound K expresses its pharmacological effects *in vivo*.[14–18] Thus, hydrophilic constituents of herbal medicines may be dependent on their metabolic activity of intestinal microflora. The *β*-glucosidase produced by intestinal microflora may play an important role in the pharmacological actions of ginseng constituents, particularly ginsenoside Rb1. Therefore, to understand the role of intestinal microflora in expressing pharmacological activity of ginsenoside Rb1, we measured fecal *β*-glucosidase and ginsenoside Rb1-metabolic activities of 148 human feces. The fecal *β*-glucosidase and ginsenoside Rb1-metabolic activities between individuals varied significantly, likely the previously reported.[19,22] However, the activity was

Table 6. K_m and V_{max} of Recombinant *β*-D-Glucosidase

Enzyme	K_m (mM)				V_{max} (*μ*mol/min/mg)			
	pNPG	Loganin	Arctiin	Rb1	pNPG	Loganin	Arctiin	Rb1
β-D-Glucosidase	0.83	1.92	1.62	4.33	56.50	11.68	20.31	4.08

These data were calculated from triplicate experiments.

not different between males and females, or between ages. Next, we investigated the relationship between the metabolic activities of these ginsenosides and β-glucosidase activity. The ginsenoside Rb1-metabolic activities of most individuals are in proportion to their β-glucosidase activities measured using *p*-nitrophenyl-β-D-glucopyranoside. Nevertheless, the ginsenoside Rb1-metabolic activities of several individuals are in proportion to their β-glucosidase activities measured using p–nitrophenyl-β-D-glucopyranoside. These results suggest that intestinal microflora produce many kinds of β-glucosidase(s), and among them, a part of β-glucosidase(s) may metabolize transform ginsenosides to the active compound(s) such as compound K. Of ginsenosides and their metabolites, compound K exhibits the most potent biological activities, such as anti-allergic and anti-tumor activities.[19,20] Based on these findings, β-glucosidase-producing intestinal bacteria may play the important role in expressing the pharmacological effects of ginsenosides.

These β-glucosidases (β-glucoside glucohydrolase, EC 3.2.1.21) are the key enzyme for carbohydrate metabolism in bacteria that are able to cleave the β-glucosidic linkages of di- and/or oligosaccharides or other glucose conjugates.[30] β-Glucosidases are widely distributed in living organisms and play pivotal roles in many biological processes, such as the degradation of cellulosic biomass,[31] cyanogenesis,[32] and the cleavage of glucosylated constituents, such as ginsenoside Rb1, in natural products.[33]

To understand the genetic and enzymatic properties of β-glucosidase of *B. longum* H-1 metabolizing ginsenoside Rb1, we cloned BglX (β-glucosidase) gene from *Bifidobacterium longum* H-1, expressed it in *E. coli* and investigated its enzymatic property.

Initially, we prepared two amino acid sequences from *B. longum* sp. The β-glucosidase gene cloned from H-1 consisted of 2364 bp, with 787 predicted amino acid residues, and the approximate molecular weight was determined to be 95 kDa. The cloned H-1 β-glucosidase expressed in *E. coli* was homogenously purified by HiTrap IMAC HP chromatography. To determine the standard reaction conditions, we measured enzymatic activity at different pH, temperature, and ionic strength, and in the presence of various divalent cations. It was confirmed that rBglX was most active in 50 mM sodium phosphate buffer (pH 5.5) at 35°C. The activity of rBglX was not affected by the strength of salt, such as NaCl. On the other hand, the enzymatic activity was strongly inhibited by the presence of Cu^{2+}. With respect to optimum PH and temperature, the enzymatic properties were similar to those of other recombinant β-glucosidases from microbes, such as *B. breve*, *Bifidobacterium* sp. strain SEN, *Bacillus subtilis* and *Streptomyces coelicolor*. However, the molecular weight of H-1 rBglX was different from that of recombinant β-glucosidases from *B. breve*, *Bacillus subtilis* and *Streptomyces coelicolor* (average molecular weight of 50 kDa),[34–36] and from β-glucosidases purified from *Bifidobacterium sp.* strain SEN.[37,38] Addition of divalent cations, like Cu^{2+} strongly inhibited H-1 rBglX activity, whereas it hardly inhibited the activity of other recombinant enzyme (remaining activity was 73%). Some of the β-D-glucosidases possess broad spectrum activities, such as β-D-galacosidase and β-D-fucosidase activities including β-D-glucosidase activity.[34–36] However, the rBglX has only β-D-glucosidase activity. Furthermore, it hydrolyzes exo-type and not endo-type D-glucose moieties in natural *O*-β-D-glycosides. The enzyme was inefficient in hydrolyzing natural *C*-glycosides, such as mangiferin and puerarin.

The rBglX hydrolyzes loganin from the fructus of *Cornus officinalis*, arctiin from the fructus of *Arctium lappa* and ginsenoside Rb1 from Ginseng and then form arctiin, loganetin and ginsenoside Rd, respectively. These products are the respective metabolites biotransformed by intestinal microflora, when loganin, arctiin and ginsenoside Rb1 were orally administered.[1,26] The metabolites arctigenin and ginsenoside Rd exhibit more potent biological effects *in vitro* than their parental compounds.[37,38] However, the biological activities of loganetin, a metabolite of loganin, have not been studied. By employing rBglX, we could easily isolate loganetin and arctiin from their glycosides (yield, >85%), respectively. The recombinant β-glucosidase may hydrolyze natural product glycosides, such as daidzin and geniposide and its activity could be considered valuable in transforming natural product glycosides. To the best of our knowledge, this is the first report on the cloning, purification, and characterization of rBglX (β-D-glucosidase) from *B. longum*.

Based on these findings, ginsenoside Rb1 may be metabolized to bioactive compound(s) by exo- β-D-glucosidase(s) produced from the intestinal bacteria, such as *B. longum* H-1, and its pharmacological effects may be dependent on intestinal bacterial *exo*-β-D-glucosidase(s) activity.

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