Cloning and Expression of β-Glucuronidase from *Lactobacillus brevis* in *E. coli* and Application in Bioconversion of Baicalin and Wogonoside

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The β-glucuronidase (*GUS*) gene from *Lactobacillus brevis* ROI was cloned and expressed in *Escherichia coli* GMS407. The *GUS* gene was composed of 1,812 bp, encoding a 603-amino-acid protein belonging to glycosyl hydroxase family 2 with three conserved domains. The amino acid similarity was higher than 70% with the β-glucuronidases of various microorganisms, yet less than 58% with the β-glucuronidase of *L. gasseri* ADH. Overexpression and purification of the GUS was performed in β-glucuronidase-deficient *E. coli* GMS407. The purified GUS protein was 71 kDa and showed 1,284 U/mg of specific activity at optimum conditions of pH 5.0 and 37°C. At 37°C, the GUS remained stable for 80 min at pH values ranging from 5.0 to 8.0. The purified enzyme exhibited a half-life of 1 h at 60°C and more than 2 h at 50°C. When the purified GUS was applied to transform baicalin and wogonoside into their corresponding aglycones, 150 µM of baicalin and 125 µM of wogonoside were completely transformed into baicalein and wogonin, respectively, within 3 h.

**Keywords:** Baicalin, baicalein, β-glucuronidase, *Lactobacillus*, wogonin, wogonoside

*Scutellaria baicalensis* is a traditional herbal medicine that is used to treat inflammation, fevers, coughs, and hepatitis in China, Korea, and Japan [8]. The principle active components of *S. baicalensis* are flavonoid glycosides, such as baicalin and wogonoside, which contain glucuronic acid attached to the flavonoid backbone [8]. Following the oral administration of *S. baicalensis*, baicalin is not detected in the plasma, but the aglycone baicalein shows two peaks: after 2 to 4 h, and after 12 h [15]. Whereas the earlier peak represents the initial absorption of baicalein naturally present in *S. baicalensis*, the second peak is thought to be baicalein released from baicalin by intestinal microflora [15], implying that the uptake of baicalin and wogonoside *in vivo* can be facilitated by the intake of their corresponding aglycones (baicalein and wogonin, respectively) that can be produced by β-glucuronidase-producing microorganisms or their enzymes. Bacterial β-glucuronidase activities have already been detected in *E. coli*, *Bacteroides*, *Eubacterium*, *Ruminococcus*, and *Lactobacillus*, and genes encoding β-glucuronidase have been characterized from *E. coli*, *L. gasseri*, *Staphylococcus* sp., *Clostridium perfringens*, *S. aureus*, *Thermotoga maritina*, and *R. gnavus* [2]. However, most of these microorganisms are pathogenic bacteria or non-GRAS (generally recognized as safe) microorganisms. Accordingly, to obtain a β-glucuronidase gene from a GRAS microorganism for the construction of a safe bioconversion system for baicalin and wogonoside, this study cloned and characterized a gene encoding β-glucuronidase from a *L. brevis* strain in *E. coli* and then applied the expressed enzyme for the bioconversion of baicalin and wogonoside into baicalein and wogonin, respectively.

**Materials and Methods**

**Bacterial Strains and Plasmids**

The bacterial strains and plasmids are listed in Table 1. *L. brevis* RO1 was grown anaerobically at 37°C in an MRS medium (BD, MD, U.S.A.) containing 0.05% l-cysteine HCl (Sigma). The *E. coli* β-glucuronidase-deficient strain, GMS407, was obtained from the National BioResource Project (NBRP, Japan). The *E. coli* strains were grown aerobically at 37°C in a Luria–Bertani medium (BD, MD, U.S.A.), and ampicillin (50 mg/ml) was added when needed.
Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. brevis RO1</td>
<td>β-Glucuronidase-producing Lactobacillus brevis</td>
<td>This study (KFCC*11424P)</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>F2 f80lacZD15M1 Di(lacZYA-argF)U169 endA1 recA1 hsdR17(rK2 mK1) deoR</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>E. coli GMS407</td>
<td>F+ zde-261::Tn5 argE3 lacY1 galK2 manA4 mtl-1 tsx-29 supE44 uidA1 β-glucuronidase-negative strain</td>
<td>NBRP</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBADNH</td>
<td>araBAD promoter region, initiation ATG, Polyhistidine tag, Xpress epitope, enterokinase recognition site, multiple cloning site, rrnB transcription termination region, ampicillin ORF, pBR322 origin, AraC ORF</td>
<td>[6]</td>
</tr>
<tr>
<td>pBADNH-GUS</td>
<td>pBADNH containing β-glucuronidase gene from L. brevis RO1</td>
<td>This study</td>
</tr>
</tbody>
</table>

*KFCC: Korean Federation of Culture Collection.

Cloning and Sequence Analysis of β-Glucuronidase from L. brevis RO1

To amplify the β-glucuronidase gene from the genome of L. brevis RO1, the PCR primers (forward/Sacl site is underlined: AAAGAGC TCAAGTATACCAATGGAAACG; reverse/XhoI site is underlined: GGGGCTTAGACTTTTTTATATAAATGCGGAATATTC) were designed based on the β-glucuronidase gene sequence obtained from the annotated genome data of L. brevis ATCC 367 [13]. The genomic DNA of L. brevis RO1 was isolated using GeneReleaser (Bioventures Inc., TN, U.S.A.) according to the manufacturer’s protocol, and used as the template for the PCR [conditions: 98°C, 10 s denaturation; 48°C, 30 s annealing; 72°C, 3 s extension; 35 cycles, LA Taq polymerase (TAKARA, Shiga, Japan)]. The PCR product was digested with Sacl and XhoI, ligated with Sacl–XhoI-digested pBADNH [6], and transformed into E. coli DH5α cells using the method of Sambrook et al. [17]. The recombinant plasmid was named pBADNH-GUS. The nucleotide sequences were determined on both strands using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 377 sequencer (Perkin-Elmer, CA, U.S.A.). The DNA and amino acid sequence data analyses were then performed using the DNASIS and PROSIS programs (HITACHI Software Engineering Co., Japan), respectively. A homology search was conducted using the World Wide Web server for BLAST searching maintained at the National Center for Biotechnology Information [1]. A multiple sequence alignment of related amino acid sequences was performed using the CLUSTAL V program [7].

Expression and Purification of β-Glucuronidase from E. coli

For the expression and purification of the β-glucuronidase (GUS), the pBADNH-GUS plasmid was transformed into the β-glucuronidase-negative E. coli strain GMS407 (NBRP, Japan). The E. coli GMS407 (pBADNH-GUS) was then incubated in 300 ml of a Luria–Bertani medium containing 100 µg/ml ampicillin at 37°C for 2.5 h, a final concentration of 0.2% arabinose was added to induce the GUS, and the cells were incubated for an additional 5 h. The β-glucuronidase from E. coli GMS407 (pBADNH-GUS) was purified using QiAexpress Ni-NTA Fast Start (QIAGEN, Germany) according to the manufacturer’s protocol with minor modifications. The cells were then disrupted by sonication (conditions: pulse-on 1 s, pulse-off 1 s, 4°C, 8 min), finally resulting in 1 ml of the purified protein fraction. The protein concentration was measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Western Blot Analysis of His-GUS

The purified GUS from GMS407 was separated on a SDS–PAGE gel and electrophoresed onto a nylon membrane to perform a Western blot analysis. The β-glucuronidase activity was measured based on the rate of release of p-nitrophenol from p-nitropheno[y]-β-d-glucuronide (PNPG) (Sigma). Eighty µl of the cell extract was warmed to 37°C, and 20 µl of 5.0 mM PNPG then added. At appropriate time intervals, the reaction was stopped by adding 100 µl of 1.0 M Na2CO3. The optical density measured at 405 nm. One unit of activity was defined as 1 nM of p-nitrophenol liberated per minute per milligram of protein. Each value is the mean of at least three independent measurements.

To examine the effects of pH and temperature on the GUS of L. brevis RO1, the cells were washed twice with a 0.1 M phosphate buffer for pHs 5–8 and a 0.1 M acetate buffer for pH 4, and then resuspended in the same buffer and disrupted by sonication. The cell extract was prepared by centrifugation at 15,000 ×g for 30 min and kept on ice until the assay. The cell extract was then warmed to 37–70°C, 80 µl added to 20 µl of prewarmed 0.5 mM PNPG, and the mixture incubated for 20 min at each temperature. The reaction was stopped by adding 100 µl of 1.0 M Na2CO3. For the GUS isolated from E. coli GMS407, 1 µl of purified GUS was mixed with 79 µl of 0.1 M phosphate buffer at different pHs and the same assay procedure followed. The enzyme stability of the purified GUS from E. coli GMS407 at different pH values was determined by measuring the remaining activity after incubation at 37°C in each pH buffer. Meanwhile, the enzyme stability at different temperatures was determined by measuring the remaining activity after incubation at pH 5.0 at each temperature.

Bioconversion of Baicalin and Wogonoside

One hundred fifty µM of baicalin (Sigma, U.S.A.) and 125 µM of wogonoside (purified by the current authors) were dissolved in a 0.1 M acetate buffer (pH 5.0), mixed with the same volume of purified enzyme (10 µg/ml), and incubated at 37°C with shaking. A crude cell extract of E. coli GMS407 transformed with pBADNH was
Table 2. HPLC conditions for analysis of baicalin and wogonoside.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (min)</th>
<th>Acetonitrile (%)</th>
<th>Water (%) (0.02% phosphoric acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalin</td>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>52.8</td>
<td>47.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Wogonoside</td>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

used as the negative control. At each time point, a reaction sample was taken and mixed with the same volume of butyl alcohol. The mixture was then centrifuged at 17,900 ×g for 5 min and analyzed using HPLC with a flow rate of 1.0 ml/min, absorbance of 254 nm, and C18 column (Alltima HP C18, HL 5U; Alltech, Italy).

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of β-Glucuronidase from L. brevis RO1

Baicalin and wogonoside are known to be hydrolyzed into baicalein and wogonin by intestinal microflora, thereby enhancing their uptake [12]. For this reason, several approaches have been taken to convert baicalin and wogonin using β-glucuronidase or microorganisms that produce this enzyme. Jiang et al. [10] attempted to immobilize β-glucuronidase and transform baicalin into baicalein. However, a relatively high amino acid sequence homology was shown with Carnobacterium sp. AT7 (62% identity, 78% similarity, gi:163790512), C. perfringens strains (NCTC 8239: 54% identity, 72% similarity; str. 13CPE, str. F4969 [18]), Str. xylosus (54% identity, 69% similarity, [4]), Str. haemolyticus JCSC1435 (52% identity, 69% similarity, [19]), Streptococcus pyogenes MGAS10270 (51% identity, 69% similarity, gi:94990683), Haemophilus somnus 129PT (51% identity, 69% similarity [3]), and Str. equi subsp. zooepidemicus (52% identity, 68% similarity [11]). In addition, several highly conserved domains were detected when multiple alignments were performed (Fig. 2). Islam et al. [9] previously identified Glu\(^{140}\) as the nucleophile residue, Glu\(^{151}\) as the acid–base residue, and Tyr\(^{184}\) as involved in the active site in the human β-glucuronidase. These residues were also conserved in the GUS of L. brevis RO1 at 509, 415, and 471, respectively (Fig. 2).

Expression and Characterization of β-Glucuronidase from L. brevis RO1 in E. coli

When pBADNH-GUS was transformed into β-glucuronidase-negative E. coli GMS407, the β-glucuronidase activity
expressed and detected in the cell-free extract of the transformed *E. coli* GMS407 was 50-fold higher than that in the wild-type *L. brevis* RO1 strain (data not shown). The his-tag-purified β-glucuronidase obtained from the transformed *E. coli* GMS407 migrated around 71 kDa by SDS-PAGE (Fig. 3A) and a Western blot analysis (Fig. 3B). The highest specific activity (1,284 U/mg) of the purified β-glucuronidase was achieved at pH 5.0 and 37°C (Fig. 4), but decreased quickly above pH 7.0. At 37°C, the purified β-glucuronidase remained stable for 80 min at pH values ranging from 5.0 to 8.0 (Fig. 5A). The purified enzyme exhibited a half-life of 1 h at 60°C and greater than 2 h at 50°C (Fig. 5B). Therefore, these results indicate that the purified enzyme was stable and more active at acidic pHs and at temperatures up to 60°C. In contrast, the β-glucuronidase from *L. gasseri* ADH showed its optimum activity at 65°C, pH 6.0 [16], whereas the β-glucuronidase from *Str. equi* subsp. *zoopiepidemicus* showed its optimum activity at 52°C, pH 5.6 [11].

**Bioconversion of Baicalein and Wogonoside**

The purified β-glucuronidase obtained from *E. coli* GMS407 harboring pBADNH-GUS completely transformed 150 µM
of baicalin and 125 µM wogonoside into baicalein and wogonin, respectively, within 3 h at 37°C, pH 5.0 (Fig. 6).

Meanwhile, the crude cell extract of *E. coli* GMS407 harboring pBADNH did not show any transformation of baicalin or wogonoside (Fig. 6).

In summary, baicalin and wogonoside were successfully transformed into their corresponding aglycones when using the purified β-glucuronidase from recombinant *E. coli* GMS407 harboring the GUS from *L. brevis*.

Acknowledgment

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References


