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# 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* RO1 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 hydrolase 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*, *Bacteriodes*, *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.

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**Table 1.** Bacterial strains and plasmids.

| Strain or plasmid | Description | Reference |
|---|---|---|
| Bacterial strains | | |
| *L. brevis* RO1 | β-Glucuronidase-producing *Lactobacillus brevis* | This study (KFCC[a]11424P) |
| *E. coli* DH5α | F2 f80d*lacZ*D*M15* D(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(rK2 mK1) *deoR* | Gibco-BRL |
| *E. coli* GMS407 | $F^-$ zdc-261::Tn5 argE3 lacY1 galK2 manA4 mtl-1 tsx-29 supE44 uidA1 β-glucuronidase-negative strain | NBRP |
| Plasmids | | |
| pBADNH | *ara*BAD promoter region, initiation ATG, Polyhistidine tag, Xpress epitope, enterokinase recognition site, multiple cloning site, *rrn*B transcription termination region, ampicillin ORF, pBR322 origin, AraC ORF | [6] |
| pBADNH-GUS | pBADNH containing β-glucuronidase gene from *L. brevis* RO1 | This study |

[a]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/SacI site is underlined: AAAGAGCTCATGTTATATCCAATGGAAACAG; reverse/XhoI site is underlined: GGGTCTAGACTATTTTTTATAATTAAAGTCCGGAATATTC) 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 SacI and XhoI, ligated with SacI–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 QIA*express* 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 electrotransferred to a nylon membrane to perform a Western blot analysis, using a primary 6× His monoclonal antibody (BD Bioscience, MD, U.S.A.) and secondary mouse antibody to confirm the expression of His-GUS in GMS407. A cell extract of strain GMS407 harboring pBADNH was used as the negative control for the Western blot analysis.

**Enzyme Assays**

The β-glucuronidase activity was measured based on the rate of release of *p*-nitrophenol from *p*-nitrophenyl-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 $Na_2CO_3$, and 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 mM 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 $Na_2CO_3$. For the GUS isolated from *E. coli* GMS407, 1 μl of purified GUS was mixed with 79 μl of a 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 mM 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.

| Substrate | Time (min) | Acetonitrile (%) | Water (%) (0.02% phosphoric acid) |
|---|---|---|---|
| Baicalin | 0 | 10 | 90 |
| | 35 | 52.8 | 47.2 |
| | 50 | 10 | 90 |
| Wogonoside | 0 | 10 | 90 |
| | 45 | 65 | 35 |
| | 70 | 10 | 90 |

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, whereas Zhang *et al*. [20] purified baicalin β-D-glucuronidase from the root of *S. viscidula* Bge to transform baicalin into baicalein. Moreover, *L. brevis* RO1 isolated from *kimchi*, a traditional fermented food made of Chinese cabbage, shows a relatively high β-glucuronidase activity and has been used to clone the gene encoding this enzyme (data not shown). There has also been one report on the cloning and characterization of β-glucuronidase from lactic acid bacteria, which used *L. gasseri* ADH [16]. However, the generation of a PCR product based on the β-glucuronidase sequence of *L. gasseri* ADH was not successful (data not shown). In the present study, a 1,800-bp PCR product (Fig. 1) covering the coding region of β-glucuronidase was obtained from the chromosome of *L. brevis* RO1 using PCR primers based on the annotated β-glucuronidase gene of *L. brevis* ATCC 367 [13]. This product was then cloned as Hig-tag fused at its N terminus into the expression vector pBADNH to construct pBADNH-GUS. A sequence analysis revealed that the cloned gene was composed of 1,812 bp with a 45% G+C content (GenBank Accession No. FJ597974), which is consistent with the general GC-content of *L. brevis* (46.4%, [5]). The predicted molecular mass and pI were determined to be 69 kDa and 5.77, respectively (http://www.scripps.edu/~cdputnam/protcalc.html). A conserved domain search using the method of Marchler-Bauer *et al*. [14] revealed that the cloned gene belongs to glycosyl hydrolase family 2 and contains three conserved domains: a TIM barrel domain (amino acid residues 278–596), sugar-binding domain (amino acids 9–180), and immunoglobulin-like β-sandwich domain (amino acids 182–276) [14]. BlastN and BlastP also showed a 99% identity (1803/1812 nt, 601/603 aa) with the β-glucuronidase of *L. brevis* ATCC 367. As anticipated from the failed PCR amplification of the β-glucuronidase gene of *L. gasseri* ADH, low nucleotide and amino acid sequence homologies were exhibited with *L. gasseri* ADH (BlastP: 39% identity, 58% similarity). 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]), *Sta. xylosus* (54% identity, 69% similarity, [4]), *Sta. 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 additon, several highly conserved domains were detected when multiple alignments were performed (Fig. 2). Islam *et al*. [9] previously identified $Glu^{540}$ as the nucleophile residue, $Glu^{451}$ as the acid–base residue, and $Tyr^{504}$ 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).

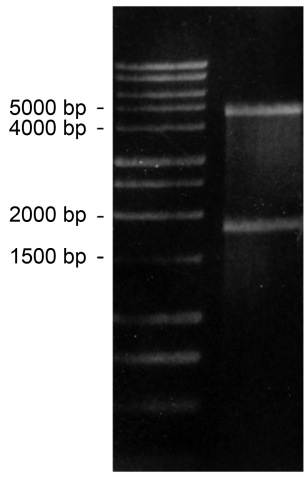


**Fig. 1.** Electrophoretogram of pBADNH-GUS after digestion using *Sac*I and *Xho*I.
The upper and lower bands correspond to pBADNH (4.6 kb) and the 1.8-kb PCR product containing the β-glucuronidase from *L. brevis* RO1, respectively.

### 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

Glu$^{451(415)}$ Tyr$^{504(471)}$ Glu$^{540(509)}$

```
I    RDKNHASVVMWSISNEAANFSKGAYEYFKP---RCLALADVIALNRYYGWYMGNGDL---PDKPIMYTEYGADTIA 516
II   RDKNHASVVMWSIANEPATHQSGSYEYFEP---KCEELANVIALNRYYGWYMQGGDL---PEKPIMYTEYGADTVP 517
III  RDKNHPCVVMWSVANEPDSDSEGAKEYFEP---KVGDIVDVLCLNRYYGWYVAGGDL---PKTPIMFTEYGADTVA 512
IV   RDKNHPCVVMWSVANEPDSDSEGAKEYFEP---KVGDIVDVLCLNRYYGWYVAGGDL---PKTPIMFTEYGADTVA 512
V    RDKNHACVVMWSIANEPASDEQGAKAYFEP---QVQDLVDVLCLNRYYGWYTQTADL---PDKPIMFTEYGADTVA 510
VI   RDKNHACVVMWSIANEPASNEKGAKAYFEP---EVQDLVDVLCLNRYYGWYTQSADL---PNKPIMFTEYGADTVA 514
VII  RDKNHPSVVMWVVANEPASHEAGAHDYFEP---EVMDLVDVICLNRYYAWYVDHGDL---PDKPILMTEYGADTLP 515
VIII RDKNHPSVVMWVVANEPASHEAGAHDYFKP---EVMDLVDVISLNRYYAWYVDHGDL---PDKPILMTEYGADTLP 508
IX   RDKNHPSVVMWVVANEPASHEEGAHEYFEP---KVMDLVDVISLNRYYAWYVDHGDL---PNKPILMTEYGADTLP 506
     *****..**** ::**. . . *: **:*--- .  :.:*:.*****.** .**---*..**: ******:.
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**Fig. 2.** Multiple alignment of β-glucuronidase amino acid sequences of several microorganisms.
Identical residues are indicated by an asterisk, conserved residues by a colon, and semiconserved residues by a dot. The three residues corresponding to human β-glucuronidase Glu$^{540}$, Glu$^{451}$, and Tyr$^{504}$ are indicated by bold type, and the numbers of corresponding amino acids in the GUS of *L. brevis* RO1 are indicated in parentheses. The origins of the β-glucuronidases are (I) *L. brevis* RO1, (II) *Carnobacterium* sp. AT7, (III) *C. perfringens* NCTC 8239, (IV) *C. perfringens* str. 13CPE, (V) *Sta. xylosus*, (VI) *Sta. haemolyticus* JCSC1435, (VII) *Str. pyogenes* MGAS10270, (VIII) *Haemophilus somnus* 129PT, and (IX) *Str. equi* subsp. *zooepidemicus.*

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. *zooepidemicus* showed its optimum activity at 52°C, pH 5.6 [11].

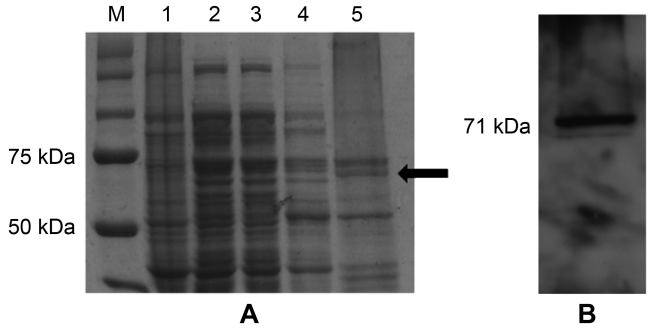


**Fig. 3.** SDS–PAGE (**A**) and Western blot analysis (**B**) of β-glucuronidase expressed and purified from *E. coli* GMS 407 harboring pBADNH-GUS.
**A**. M: protein standard; 1: cell extract before induction; 2: cell extract after arabinose induction; 3: flow-through sample; 4: sample from wash solution; and 5: purified sample. **B**. Western blot analysis of purified GUS from *E. coli* GMS407.

## Bioconversion of Baicalin and Wogonoside

The purified β-glucuronidase obtained from *E. coli* GMS407 harboring pBADNH-GUS completely transformed 150 μM

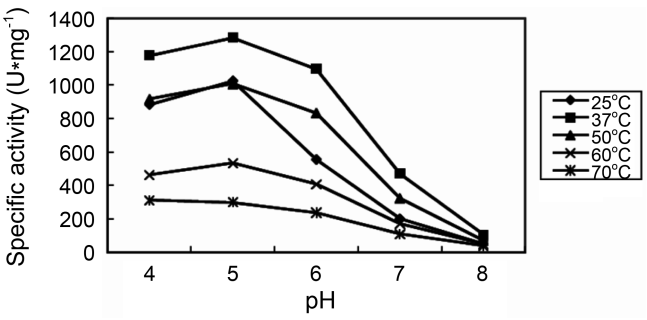


**Fig. 4.** Effects of pH and temperature on the enzyme activity of purified β-glucuronidase from *E. coli* GMS407 harboring pBADNH-GUS.

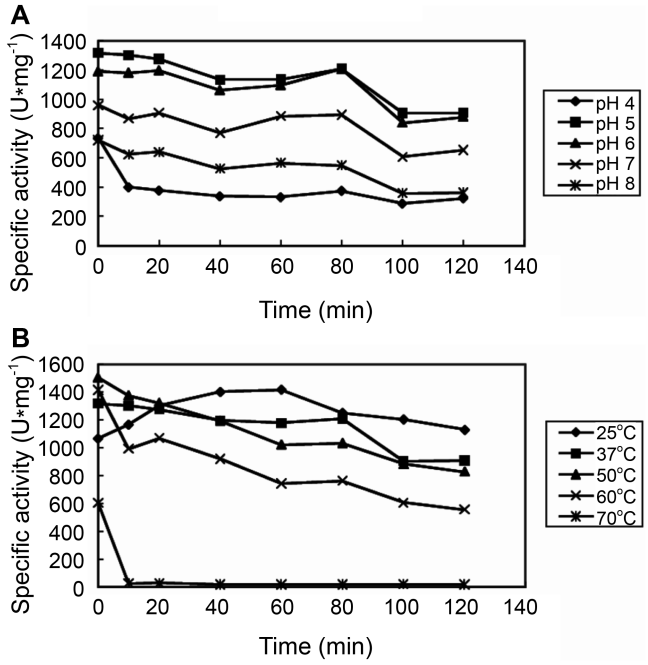


**Fig. 5.** Stability of purified β-glucuronidase at various pHs and temperatures.
**A**. pH stability at 37°C. **B**. Temperature stability at pH 5.0.

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).

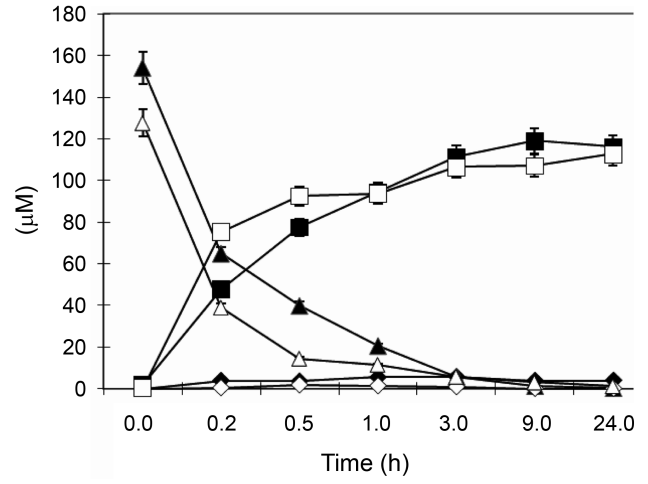


**Fig. 6.** Bioconversion of baicalin and wogonoside using crude cell extract or purified β-glucuronidase samples.
Changes in concentration of baicalin (◆) and wogonoside (◇) reacted with cell-free extract of *E. coli* GMS407 harboring pBADNH. Changes in concentration of baicalin (▲), baicalein (■), wogonoside (△), and wogonin (□) reacted with purified β-glucuronidase from *E. coli* GMS407 harboring pBADNH-GUS.

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.*

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