Research article

Production of bioactive ginsenoside Rg3(S) and compound K using recombinant Lactococcus lactis

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Background: Ginsenoside Rg3(S) and compound K (C-K) are pharmacologically active components of ginseng that promote human health and improve quality of life. The aim of this study was to produce Rg3(S) and C-K from ginseng extract using recombinant Lactococcus lactis.

Methods: L. lactis subsp. cremoris NZ9000 (L. lactis NZ9000), which harbors β-glucosidase genes (BglPM and BglBX10) from Flavobacterium johnsoniae and Paenibacillus mucilaginosus, respectively, was reacted with ginseng extract (protopanaxadiol-type ginsenoside mixture).

Results: Crude enzyme activity of BglBX10 values comprised 0.001 unit/mL and 0.003 unit/mL in uninduced and induced preparations, respectively. When whole cells of L. lactis harboring pNZBglBX10 were treated with ginseng extract, after permeabilization of cells by xylene, Rb1 and Rd were converted into Rg3(S) with a conversion yield of 61%. C-K was also produced by sequential reactions of the permeabilized cells harboring each pNZBgl and pNZBglBX10, resulting in a 70% maximum conversion yield.

Conclusion: This study demonstrates that the lactic acid bacteria having specific β-glucosidase activity can be used to enhance the health benefits of Panax ginseng in either fermented foods or bioconversion processes.

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1. Introduction

Ginseng, the root of Panax ginseng Meyer, has been used as a traditional oriental medicine to treat various diseases for more than 2,000 years [1,2]. The world ginseng market, including ginseng root and its processed products, is estimated to be worth United States (US) $2,084 million [3]. The majority of ginseng pharmacological effects are attributed to ginsenosides, which are triterpene saponins. Deglycosylated ginsenosides exhibit higher biological activity than glycosylated forms because of their smaller size, higher bioavailability, and better ability to penetrate the cell membrane [4].

Among various ginsenosides, Rg3(S), which is deglycosylated from Rd, exhibits a wide spectrum of pharmacological properties, including anticancer effects, as it has been shown to inhibit tumor cell invasion and metastasis [5]. Another attractive minor ginsenoside is compound K (C-K), which is deglycosylated from F2. While most ginsenosides are poorly absorbed from the gut, C-K is absorbed and therefore exhibits higher biological activities. Recently, in vitro and in vivo studies showed that C-K has multiple pharmacological activities, including anticarcinogenic, anti-inflammatory, antiallergic, anti-diabetic, anti-aging, and hepatoprotective effects [6]. C-K is used as the principal ingredient in some skin care products, because C-K is an effective antiwrinkle and antiaging agent for the skin [7]. However, the Rg3(S) and C-K content in ginseng is relatively low, and isolation of these valuable compounds from natural products is difficult [8,9]. Thus, efficient
methods to produce Rg3(S) and C-K are needed for their application as active pharmacological substances. Production of ginsenosides has been carried out by various methods such as heat treatment [10], alkaline cleavage [11], mild acid hydrolysis [12], enzymatic conversion [13], and microbial conversion [14]. However, physicochemical methods show poor selectivity and often produce side reactions. By contrast, the enzymatic method is regarded as desirable for the production of minor ginsenosides because of its specificity in hydrolyzing sugar moieties at particular positions. *Escherichia coli* is the most frequently used microorganism for the expression of heterologous enzymes, because the system is well characterized, genetic information is available, and the cells have a fast growth rate along with the potential for high-density cultivation in inexpensive media [15]. However, a major disadvantage of this system is the concern about the potential for high-density cultivation in inexpensive media [15].

2. Materials and methods

2.1. Materials

Bacterial strains, plasmids, and primers used in this study are listed in Table 1. *L. lactis* NZ9000 and the pNZ8008 plasmid were used as the host and gene expression vector, respectively. *L. lactis* NZ9000 was grown in M17 medium (Difco, Detroit, MI, USA) supplemented with 0.5% glucose (GM17) at 30°C. *E. coli* MC1061 (MobiTec, Goettingen, Germany) was grown in Luria-Bertani medium at 37°C under shaking conditions and used as the cloning host. For the selection of *E. coli* or *L. lactis* transformants, chloramphenicol (10 μg/mL) was added to the Luria-Bertani or GM17 medium. Gene expression in *L. lactis* was induced by nisin, which was prepared as follows: 2.5% nisin powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.05% acetic acid to obtain a final nisin concentration of 1 ng/mL. The protopanaxadiol-type ginsenoside mixture (PPDGM) was extracted from a ginseng root. The standard compounds of ginsenosides, Rb1, Rd, Rg3(S), F2, and C-K (> 98.0% purity), were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China).

2.2. Construction of pNZBglBX10 and transformation into *L. lactis*

Based on sequence information in the National Center for Biotechnology Information genome database (http://www.ncbi.nlm.nih.gov), the BglBX10 gene from *F. johnsoniae* UW101 was amplified from pGEX-bglBX10 by polymerase chain reaction (PCR) using the specific primer set indicated in Table 1 [8]. The amplified fragment was inserted into corresponding sites in the expression plasmid pNZ8008 (4,953 bp), which was linearized by digestion with EcoRI and HindIII, resulting in pNZBglBX10 (5,572 bp). A schematic diagram of the recombinant plasmid pNZBglBX10 construction is shown in Fig. S1. For the transformation step, the recombinant pNZBglBX10 plasmid was electroporated into *L. lactis* NZ9000 as previously described [16]. The transformed cells were selected using the GM17 agar with chloramphenicol.

In addition, *L. lactis* harboring pNZBgl was used to produce C-K. This recombinant strain was constructed in our previous study by
cloning the nucleotide sequence of the BglPm gene from *P. mucilaginosus* into pNZ8008 [16].

### 2.3. Expression and purification of recombinant pNZBglBX10

Recombinant *L. lactis* harboring pNZBglBX10 was cultivated in GM17 medium containing 10 μg/mL chloramphenicol at 30°C. The precultured cells (4%) were inoculated into 100 mL of fresh medium and cultured until the optical density (OD600nm) reached 0.4. Expression of the recombinant gene was induced with 1 ng/mL nisin, while uninduced cultures were used as a control. Then, the cells were grown for an additional 3 h at 30°C in 150 μL of the 50mM sodium phosphate buffer (pH 7.0) containing 4mM p-nitrophenyl-β-D-glucopyranoside. One unit of activity was defined as the amount of enzyme that produced 1 μmol of p-nitrophenol in 1 min, as measured by a microplate reader at 405 nm. In addition, the effect of culture duration on enzyme activity was determined by culturing and harvesting cells at 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h, and 24 h after nisin induction. Enzyme activity was measured using the method described above. After optimization of the expression conditions, the specific activities of crude and purified β-glucosidases were also determined. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

### 2.4. Optimization of pNZBglBX10 expression in *L. lactis* and analysis of specific enzyme activity

To investigate the effect of nisin concentrations on β-glucosidase enzyme activity, *L. lactis* harboring pNZBglBX10 was induced by 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, and 50 ng/mL nisin when the OD600nm reached 0.4. The enzyme activity of crude β-glucosidases was measured using p-nitrophenyl-β-D-glucopyranoside as a substrate. The crude enzyme (150 μL) was incubated at 37°C in 150 μL of the 50mM sodium phosphate buffer (pH 7.0) containing 4mM p-nitrophenyl-β-D-glucopyranoside. One unit of activity was defined as the amount of enzyme that produced 1 μmol of p-nitrophenol in 1 min, as measured by a microplate reader at 405 nm. In addition, the effect of culture duration on enzyme activity was determined by culturing and harvesting cells at 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h, and 24 h after nisin induction. Enzyme activity was measured using the method described above. After optimization of the expression conditions, the specific activities of crude and purified β-glucosidases were also determined. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

### 2.5. Bioconversion of PPDGM into Rg3(S) using recombinant *L. lactis* cells

To produce Rg3(S), three preparations (whole cells, cell lysates, and permeabilized cells) of recombinant *L. lactis* harboring pNZBglBX10 were used. After cultivation, whole cells were harvested by centrifugation at 10,000g for 10 min at 4°C and suspended in 50mM sodium phosphate buffer (pH 7.0). To prepare cell lysates, whole cells were disrupted by sonication, and the supernatant fraction was recovered after centrifugation. For permeabilized cells, 0.5% xylene was added to the whole cell preparations [17]. Then, the three types of cells (whole cells, cell lysates, and permeabilized cells, at a final concentration of 300 mg/mL) were reacted with 1% PPDGM in 50mM sodium phosphate buffer (pH 7.0) for 24 h. Samples were taken at 0 h, 6 h, 12 h, and 24 h intervals and centrifuged (10,000g, 2 min, 4°C) after boiling for 5 min. Both supernatant and residue fractions were extracted with 50% ethanol and the sum of the two fractions was used for analysis.

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**Fig. 1.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of BglBX10 expressed in *Lactococcus lactis*. Control, *L. lactis* harboring pNZBglBX10 without induction; pNZBglBX10, *L. lactis* harboring pNZBglBX10 with nisin induction. E, elution fraction after Ni-NTA purification; I, insoluble fraction of cell extracts; M, molecular weight marker; S, soluble fraction; T, total fraction.

**Fig. 2.** Changes in β-glucosidase activity of recombinant *Lactococcus lactis* harboring pNZBglBX10 (A) at different concentrations (0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, and 50 ng/mL) of nisin and (B) at different culturing times (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h, and 24 h) after induction by 1 ng/mL nisin.
2.6. Bioconversion of major ginsenosides to C-K using recombinant L. lactis harboring pNZBgl and/or pNZBglBX10

To produce C-K, recombinant strains of L. lactis harboring pNZBgl [16] and/or pNZBglBX10 were used. Three methods were employed for the synthesis of ginsenoside C-K. The first method was a combined mode with reactions of mixed cells; after cultivation, the whole cells of both L. lactis (pNZBgl) and L. lactis (pNZBglBX10) were harvested (final concentration of cells: 100 mg/mL), and they were allowed to simultaneously react in 50mM sodium phosphate buffer (pH 7.0) with 1% PPDGM and 0.5% xylene for 36 h. The second method was a sequential mode in which two types of cells reacted one by one; L. lactis harboring pNZBgl (100 mg/mL) reacted in 50mM sodium phosphate buffer (pH 7.0) with 1% PPDGM and 0.5% xylene for 24 h, and when the ginsenoside Rb1 was almost fully converted to F2, L. lactis harboring pNZBglBX10 (100 mg/mL) was also added for an additional reaction for 48 h. The third method was a dual-plasmid mode, with the reaction of a recombinant cell harboring two plasmids (pNZBgl and pNZBglBX10); the two plasmids were simultaneously transformed into L. lactis which was confirmed by colony PCR using two specific primers for each β-glucosidase genes (BglPm and BglBX10) and the resulting recombinant cells (200 mg/mL) were reacted in the same conditions. Samples were taken at intervals of 0 h, 12 h, 18 h, 24 h, and 36 h for the combined mode and dual-plasmid mode, and at 0 h, 12 h, 18, 24 h, 36 h, 42 h, 48 h, 60, and 72 h for the sequential mode.

2.7. HPLC analysis

Ginsenoside [Rb1, Rd, F2, Rg3(S), and C-K] concentrations were quantified using an Agilent 1260 Infinity HPLC (Agilent Technology, Santa Clara, CA, USA) system equipped with a ZORBAX SB-C18 column (4.6 mm × 150 mm). Acetonitrile (Solvent A) and water (Solvent B) were used as the mobile phases. Gradient elution was performed, beginning with 70% Solvent A and 30% Solvent B for 5 min, and progressing to 70% Solvent A and 30% Solvent B for 5–15 min; 43% Solvent A and 57% Solvent B for 15–25 min; 30% Solvent A and 70% Solvent B for 25–30 min; and 30% Solvent A and 70% Solvent B for 30–40 min. The flow rate of the mobile phase was 0.8 mL/min, and it was monitored at 203 nm absorbance using a UV spectrophotometric detector. The bioconversion yields of the ginsenosides Rg3(S) and C-K were calculated as follows: the conversion yield of Rg3(S) (%) = \(\frac{D_{Rg3(S)}}{D_{Rb1} + D_{Rd}} \times 100\), and the conversion yield of C-K (%) = \(\frac{D_{C-K}}{D_{Rb1} + D_{Rd}} \times 100\).

Table 2

| Lactococcus lactis (pNZBglBX10) | Step | Volume (mL) | Activity (unit/mL) | Total activity (unit) | Protein (mg/mL) | Specific activity (unit/mg) | Yield (%) | Purification fold |
|---------------------------------|------|-------------|--------------------|-----------------------|-----------------|-----------------------------|-----------|------------------|
| Uninduced                      | Crude enzyme | 100 | 0.001 | 0.1 | 1.5 |                     |           |                  |
| Induced                        | Crude enzyme | 100 | 0.003 | 0.3 | 1.3 | 0.002 | 100 | 1                |
|                               | Ni-NTA purification | 100 | 0.002 | 0.2 | 0.1 | 0.02  | 67  | 10               |

1) One unit of activity corresponds to the production of 1 μmol p-nitrophenyl (PNP)/min

Table 3

| Samples | Concentration (mM) | Yield (%) |
|---------|-------------------|-----------|
|         | Ginsenoside Rb1    | Ginsenoside Rd | Ginsenoside Rg3(S) |
|         | 0 h     | 24 h       | 0 h | 24 h | 0 h | 24 h |
| Control | 3.3 ± 0.03 | 2.16 ± 0.05 | 0.14 ± 0.08 | 0.14 ± 0.08 | 0 |
| Whole cells | 0.19 ± 0.02 | 0.28 ± 0.04 | 2.34 ± 0.04 | 44 |
| Cell lysates | 0.94 ± 0.00 | 3.70 ± 0.01 | 2.40 ± 0.01 | 58 |
| Permeabilized cells | 0.22 ± 0.05 | 0.73 ± 0.02 | 2.88 ± 0.05 | 61 |

The values in the table are averages with standard deviations determined from three independent experiments

1) Conversion yield (%) = \(\frac{D_{Rg3(S)}}{D_{Rb1} + D_{Rd}} \times 100\)

Fig. 3. Schematic presentation of the transformation pathways used to produce (A) Rg3(S) and (B) C-K and the structures of ginsenosides.
3. Results and discussion

3.1. Construction of recombinant L. lactis

There have been many previous reports that low levels of heterologous expression were caused by differences in synonymous codon usage between the expression and natural hosts, as well as because of the accumulation of rare codons during expression of the heterologous protein [18,19]. To determine whether heterologous β-glucosidase could be expressed at a high level in L. lactis, the codon usage was analyzed. For this, the frequency of each codon (count per thousand) of BglBX10 gene and L. lactis genome were analyzed at http://www.bioinformatics.org/sms2/codon_usage.html and http://www.kazusa.or.jp/codon/, respectively. The codon usage was found to be similar in the two cases, except for CCG (proline), suggesting that codon usage differences would not significantly affect the β-glucosidase expression level. Therefore, the recombinant plasmid pNZBglBX10 was constructed by inserting the native BglBX10 gene and it was transformed into the host, L. lactis NZ9000.

3.2. Expression and characterization of the BglBX10 enzyme

To measure the expression of the BglBX10 gene in L. lactis, SDS-PAGE analysis was conducted. As shown in Fig. 1, bands corresponding to the β-glucosidase protein were present in both the total and soluble fractions of the nisin-induced cell samples, whereas no bands were detected in the uninduced samples. The protein was purified to homogeneity using an Ni-NTA affinity chromatography column. The molecular weight of the recombinant protein was estimated to be 89 kDa by SDS-PAGE, which was similar to the theoretical value (89.3 kDa) calculated from its amino acid sequence.

To investigate the effects of different nisin concentrations, L. lactis harboring pNZBglBX10 was induced with 0.5 ng/mL, 1 ng/mL, 10 ng/mL, 20 ng/mL, and 50 ng/mL nisin in GM17 medium supplemented with chloramphenicol. As shown in Fig. 2A, β-glucosidase activity in the cytoplasmic fraction was similar (9 munit/mL/OD600nm) with nisin concentrations ranging from 0.5 ng/mL to 10 ng/mL. However, it decreased by half at higher nisin concentrations (20 ng/mL and 50 ng/mL) due to the inhibitory effect of nisin on microbial cell growth. Therefore, in the next part of this study, 1 ng/mL of nisin was used to induce enzyme expression in L. lactis. In addition, the effect of the postinduction period on enzyme activity was determined. As shown in Fig. 2B, the highest enzyme activity (7.5 munit/mL/OD600nm) was detected at 4 h after nisin induction; thereafter, the total enzyme activity decreased.

In order to measure the specific activities of crude and purified preparations of β-glucosidase expressed in L. lactis harboring pNZBglBX10, cell-free extract was obtained after induction. The activity values of the BglBX10 enzyme in uninduced and induced cultures were 0.001 unit/mL and 0.003 unit/mL, respectively. The Ni-NTA-purified enzyme had an activity value of 0.002 unit/mL (Table 2). The specific activities of β-glucosidase in the crude extract and purified fraction were 0.002 units/mg and 0.02 units/mg, respectively.

3.3. Production of Rg3(S) using different preparations of recombinant cells

To produce Rg3(S) from PPDGM, purified from ginseng root, three different preparations of the recombinant cells (L. lactis harboring pNZBglBX10), namely whole cells, cell lysates, and permeabilized cells, were used. The concentrations of Rb1 and Rd in the 1% PPDGM solution were approximately 3.4mM and 2.4mM, respectively. As shown in Table 3, Rb1 was completely consumed by all three preparations, whereas no change was observed in the control fraction (uninduced cells). The ginsenoside Rg3(S) was produced after 5 h, and most conversions were completed within 24 h. A small amount of Rd, another major PPDGM component, remained after a 24 h reaction. The bioconversion yields of whole cells, cell lysates, and permeabilized cells were 44%, 58%, and 61%, respectively.
These results indicate that the enzyme was located mostly in the cytoplasm and could be released into the culture medium by manipulations such as sonication or xylene treatment. The use of whole cells has several advantages over the use of purified enzymes in many industrial bioconversion processes. However, reaction rates in whole cells are often very low because the cell envelope presents a permeability barrier for substrates and products [20]. To reduce the permeability barrier and obtain more enzymatically active whole cell preparations, cells can be permeabilized by ethanol or toluene. Permeabilization is an economical, easy, convenient, and safe process for enzymatic bioconversion and product formation [21]. In addition, the ginsenoside C-K was also produced during the bioconversion reactions. However, yields of bioconversion of PPDGM into C-K by whole cells, cell lysates, and permeabilized cells were very low (1%, 7%, and 5%, respectively), and BglBX10 could convert F2 into C-K when there was enough F2 (Fig. 3B). In the addition of the BglBX10 enzyme, F2 was almost converted into C-K within 12 h, and a small amount of Rg3(S) was synthesized and C-K were 25%, 5%, and 70%, respectively, in the sequential mode, with the bioconversion yield of C-K being the highest. Because F2 was fully synthesized as a final product in the dual-plasmid mode, the bioconversion yield of F2 was almost 100%.

4. Conclusions

This study demonstrates that lysated or permeabilized L. lactis expressing the β-glucosidase gene can be used to efficiently produce the compound Rg3(S) from ginseng extract. The combined use of the two glucosidases enabled the efficient production of C-K. To our knowledge, this is the first report of a method to produce Rg3(S) and C-K with high conversion yields, using a system based on LAB. This food-grade microbial cell factory can be used to enhance the health benefits of Panax ginseng, in either fermented foods or bioconversion processes.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgr.2017.04.007.

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