Production of ginsenoside F1 using commercial enzyme Cellulase KN

Yu Wang1,2, Kang-Duk Choi3, Hongshan Yu1, Fengxie Jin1,**, Wan-Taek Im2,3,*

1 College of Biotechnology, Dalian Polytechnic University, Ganjingzi-qu, Dalian, PR China
2 Department of Biotechnology, Hankyong National University, Anseong, Korea
3 Genomic Informatics Center, Graduate School of Future Convergence Technology, Hankyong National University, Anseong, Korea

**Corresponding author. Department of Biotechnology, Dalian Polytechnic University, Qinggong-yuan Number 1, Ganjingzi-qu, Dalian 116034, PR China.

E-mail addresses: fxjin@dlpu.edu.cn (F. Jin), wandra@hknu.ac.kr (W.-T. Im).

1. Introduction

Ginseng, a well-known medicinal plant, has not only been used as a traditional medicine in East Asia for thousands of years, but has also gained recognition in the past decade in the West for its beneficial uses [1–3]. Many previous reports have shown that ginseng has extensive pharmacological and therapeutic utility. Ginsenosides, the major components of ginseng, appear to be responsible for the principal biological and pharmacological activities underpinning anticancer, antioxidant, anti-inflammatory, and anti-inflammatoty effects [4–10]. Based on the structure of the aglycon and the dammarane skeleton, ginsenosides can be categorized as protopanaxadiol, protopanaxatriol (PPT), and oleanane saponins [11]. The PPT-type ginsenosides are further identified by the number of sugar moieties and the position of a glycosidic bond at the aglycon C6 and C20. After oral intake of ginseng, the major ginsenosides are hydrolyzed into the more active minor ginsenosides through human intestinal digestion, and are further absorbed into the blood. Therefore, conversion of the major ginsenosides, which account for > 80% of the total ginsenosides, to the highly active minor ginsenosides may have a significant impact on the pharmaceutical industry [4,5].

The minor ginsenoside F1 is present in low concentrations in Korean ginseng. It can be processed through deglycosylation by intestinal microflora from the major ginsenosides Re and Rg1; it is absorbed into the blood, where it exerts an estrogenic effect [12]. Ginsenoside F1 exhibited anticancer effect, showing strong suppression of B16 cell proliferation [13]. Furthermore, ginsenoside F1 shows antiaging and antioxidant effects, and exhibits competitive inhibition of the activity of CYP3A4 and weaker inhibition of the activity of CYP2D6 [14,15].
Ginsenoside F1 can be produced using microbes or through enzymatic processes. One example is *Fusarium moniliforme* var. *sublutinas*, which has β-glucosidase activity and has shown the ability to convert ginsenoside Rg1 into F1 [16]. Another example is commercially available naringinase from *Penicillium decumbens* [17], which has the ability to hydrolyze glucose and the rhamnose moieties at the C6 position to produce ginsenoside F1 from Re and Rg1. However, in that study, the researchers conducted only a simple enzyme presentation, without further scale-up or process engineering. Although several PPT-type ginsenoside-hydrolyzing recombinant enzymes have been constructed [18–22], the majority of these have the ability to hydrolyze the glucose moiety at the C20 position of aglycon to produce Rg2(S) or Rh1(S) from Re or Rg1, respectively, rather than the glucose moiety at the C6 position of aglycon. Among these, two enzymes were reported to have hydrolyzing activity for the glucose moiety at the C6 position of PPT aglycon to produce ginsenoside F1 [19,21]. However, the researchers conducted only a simple enzyme characterization, without further scale-up or process engineering.

The experiment in this study was designed to overcome the above disadvantages and meet the industrial demand for mass production of ginsenoside F1 as a food-grade health supplement. From several commercial enzymes capable of transforming ginsenosides, we selected Cellulase KN on the basis of its high activity for transforming ginsenosides Re and Rg1 into ginsenoside F1. Treatment of a PPT-type ginsenoside mixture (PPTGM) composed of Re and Rg1 mainly with Cellulase KN followed by purification yielded 10 g-scale F1 with high purity. This is the first report of 10 g-scale production of high-purity F1 by the application of commercial enzymes to a crude substrate.

2. Materials and methods

2.1. Materials

Standard-grade ginsenosides, including Rg1, Re, Rh1(S), F1, and PPT, were purchased from Nanjing Zelang Medical Technology Co., Ltd (Nanjing, China). The PPTGM was extracted from the dried roots of 4 kg of *Panax ginseng* Meyer together with 1 kg of *Panax quinquefolius*. Five kilograms of ginseng root powder was extracted with 50 L of 70% ethanol twice. The extract was filtered through filter papers and then dried using a rotary evaporator. The resultant dried powder was dissolved in water and loaded onto a glass column (100 mm L × 100 mm D) packed with Diaion HP-20 resin (Tokyo, Mitsubishi Chemical). Free sugar molecules and unwanted hydrophilic compounds from HP-20 absorbed beads were washed with 8 column volumes of water, and finally PPT-type ginsenosides were eluted by 8 column volumes of 36% ethanol. The ethanol extracts were evaporated in vacuo, and the dried residue was used as the substrate ginsenoside for F1 production. According to the HPLC analysis, the PPTGM was mainly composed of Re (245 mg/g), Rg1 (203 mg/g), Rb1 (32 mg/g), Rb2, (25 mg/g), Rb3 (53 mg/g), Rd (80 mg/g), and small amounts of other ginsenosides, and was dissolved in 50mM acetate buffer, which could solubilize the PPTGM at up to 100 mg/mL [19]. HPLC-grade methanol and acetonitrile were obtained from SK Chemical Co., Ltd (Seoul, Korea). The other chemicals used in this study were of analytical grade or better.

2.2. Screening of commercial enzymes that can transform PPT-type ginsenosides

The PPTGM was diluted in 500 µL of acetate buffer (pH 5.0) as 1% (w/v) and the same volumes of commercial enzymes—Novozym 960 (Bagsvaerd, Novozyme), Viscozyme (Novozyme), Pectinex AFPL-4 (Novozyme), Pectinex Ultra SP-L (Novozyme), Fungamyl 800L (Novozyme), and Cellulase KN (0.2 g/mL, Kagawa, Kyowa Chemical)—to be mixed for subsequent testing. The mixture was shaken aerobically at 150 rpm and 50°C for 48 h. The reactants were taken at regular intervals and analyzed via TLC or HPLC after pretreatment (see “Analytic methods” section).

2.3. Biotransformation activity of PPT-type ginsenosides using Cellulase KN

The activity of Cellulase KN was examined to determine the specificity and selectivity of conversion of ginsenosides Re and Rg1 through hydrolysis of the rhamnose and glucose moieties attached at the C6 position. Enzyme solutions at a concentration of 100 mg/mL in 100mM acetate buffer (pH 5.0) were reacted with an equal volume of Re and Rg1 solution at a concentration of 1 mg/mL in 100mM acetate buffer (pH 5.0) at 50°C. The samples were taken at regular intervals and analyzed via TLC or HPLC after pretreatment (see “Analytic methods” section).

2.4. Biotransformation optimization based on concentration of the enzyme and substrate

In order to determine the optimal conditions for biotransformation of the PPTGM using Cellulase KN, the substrate concentration of PPTGM in the reaction was optimized. The final enzyme concentration was fixed at 50 mg/mL and 100 mg/mL and reacted with the PPTGM dissolved in acetate buffer solution (pH 5.0), in order to obtain the final substrate concentrations of 5 mg/mL, 10 mg/mL, and 15 mg/mL. The six optimization reactions were performed in a 2 ml Eppendorf tube with a 1 mL working volume at 200 rpm for 48 h at 50°C. The samples were taken at regular intervals and analyzed via TLC and HPLC.

2.5. Scaled-up biotransformation of PPTGM using Cellulase KN

The scaled-up biotransformation was performed in a 10 L stirred-tank reactor (Biotron GK; Hanil Science Co. Ltd, Seoul, Korea) with a 5 L working volume at 200 rpm for 48 hours. The reaction was performed under pH 5.0 at 50°C. The reaction started with a composition of 10 mg/mL of substrate ginsenoside (PPTGM; total 50 g) as the final concentration, and 500 g of Cellulase KN was added. Samples were collected at regular intervals and analyzed by HPLC in order to determine the production of ginsenoside F1 from the PPTGM.

2.6. Purification of F1

Following the 5 L reaction of the PPTGM with Cellulase KN, the mixture was cooled at 4°C and centrifuged at 4,000 rpm for 20 min (Component R; Hanil Science Co. Ltd). The biotransformed ginsenoside F1 in the supernatant and precipitate was processed separately in order to remove the enzymes, salt, and free sugars from the reaction mixture. Ginsenoside F1 in the supernatant was purified using a Biotage SNAP flash chromatography cartridge (180 × 70 mm; Biotage, Uppsala, Sweden) packed with 340 g of octadecylsilane (ODS; ZEOprep 60 C18, 40–63 µm), based on gradient concentration elution with methanol. The precipitate was also dissolved in 3.0 L of 95% ethanol solution twice and filtered through a filter paper (Advantec, Tokyo, Japan) and evaporated in vacuo.

The resulting ginsenoside powder derived from the supernatant and precipitate was denoted as crude ginsenoside F1 and purified further using a Biotage SNAP flash chromatography cartridge (180 × 70 mm; Biotage) packed with 340 g of silica resin (230–400 mesh). The cartridge was equilibrated with chloroform, and
13.5 g of powdered crude F1 was dry-loaded into a self-packed sample cartridge twice. Elution was performed with 3 bed volumes of chloroform and 5 bed volumes of chloroform–methanol (85:15, v/v). Fractions were taken for every 340-mL elution (0.5 bed volume), and the results were analyzed by TLC. The eluent was evaporated in vacuo. The resulting powder was dissolved in 100% methanol and analyzed via HPLC.

2.7. Analytic methods

2.7.1. TLC analysis
TLC was performed using 60F254 silica gel plates (Merck, Darmstadt, Germany) with CHCl3–CH3OH–H2O (65:35:10, lower phase) as the solvent. The spots on the TLC plates were identified through comparisons with standard ginsenosides after visualisation by spraying 10% (v/v) H2SO4, followed by heating at 110°C for 5 min.

2.7.2. HPLC analysis
HPLC analysis of the ginsenosides (Re, Rd, F1, F2, Rg1, Re, C-K, F1, and PPT) was performed using an HPLC system (Younglin Co., Ltd, Seoul, Korea), with a quaternary pump, automatic injector, single-wavelength UV detector (model 730D), and Younglin’s AutoChro 3000 software for peak identification and integration. The separation was carried out on a Prodigy ODS(2) C18 column (5 μm, 150 × 4.6 mm i.d.; Phenomenex, Torrance, CA, USA) with a guard column (5 μm, 12.5 × 4.6 mm i.d.; Eclipse XDB C18). The mobile phases were acetonitrile (A) and water (B). Gradient elution started with 17% solvent A and 83% solvent B, and was then changed as follows: A from 17% to 25%, 12–20 min; A from 25% to 32%, 20–30 min; A from 32% to 55%, 30–35 min; A from 55% to 60%, 35–40 min; A from 60% to 80%, 40–45 min; A from 80% to 100%, 45–50 min; A 100%, 50–54 min; A from 100% to 17%, 54.0–54.1 min; and A 17%, 54.1–65 min. The flow rate was 1.0 mL/min; detection was performed by monitoring the absorbance at 203 nm.

3. Results

3.1. Selection of the most effective enzyme for the production of ginsenoside F1

We examined the generation of ginsenoside F1 from the PPTGM reacted with several commercial enzymes for 2 d. As shown in Table 1, ginsenoside F1 contents produced in the enzyme-treated reaction were determined by the percentages of ginsenoside F1 among the entire measurable ginsenoside peak area using an HPLC analysis. The commercial enzymes Novozym 960, Viscozyme, Pectinex AFPL-4, Pectinex Ultra SP-L, and Fungamyl 800 L (Novozyme) could not transform all of the ginsenosides Re and Rg1 to ginsenoside F1. However, Cellulase KN effectively transformed ginsenosides Re and Rg1 to ginsenoside F1. As the PPTGM has ginsenoside Re and Rg1 contents of 27.3% and 37.0%, respectively, based on the peak area, the content of ginsenoside F1 treated with Cellulase KN reached 57.9%, based on the peak area. The other ginsenosides detected were a small amount of untransformed ginsenoside Re, and protopanaxadiol-type ginsenoside F2 and compound K derived from the ginsenosides Rb1, Rb3, and Rd. As a result, among the six enzymes tested, ginsenoside F1 could be produced effectively using Cellulase KN.

3.2. Biotransformation of PPT ginsenosides using Cellulase KN

For verification of the bioconversion pathway of the two PPT ginsenosides Re and Rg1 by Cellulase KN, TLC analyses were performed at regular intervals. When Re and Rg1 (1.0 mg/mL) were used as substrates, they were biotransformed into ginsenoside F1 within 48 h by 100 mg/mL of Cellulase KN, as shown by the Rf values of the TLC analysis (Fig. 1). Bioconversion rate of ginsenoside Re by Cellulase KN is much lower than that of ginsenoside Rg1. When ginsenoside Rg1 was produced from ginsenoside Re by Cellulase KN, it was transformed into ginsenoside F1 quickly, so it hardly remained in the reaction mixture. Thus, it is concluded that Cellulase KN efficiently hydrolyzed the rhamnose and glucose moieties successively at the C6 position of Re, transforming it into ginsenoside F1.

Cellulase KN provides the functionality of cellulase and naringinase. It is generally accepted that cellulase has three main groups of cellulolytic enzymes: cellobiohydrolase, endoglucanase, and β-glucanase [23]. As β-glucanase is regarded the most suitable enzyme for hydrolysis of the β-glucosidic linkage at C6 in PPT-type ginsenosides, it is inferred that ginsenoside Rg1 is transformed into ginsenoside F1 by the action of β-glucosidase. In addition, naringinase is regarded as having α-L-rhamnogalactosidase activity against the 1→2→3→α-L-rhamnose linkage attached to the C6 hydroxyl group of aglycon in ginsenoside Re and also displays higher β-glucosidase activity toward the glucosidic linkage at C6 in ginsenoside Rg1. The above results indicated that Cellulase KN is highly effective for biotransformation of ginsenosides Re and Rg1 into ginsenoside F1.

3.3. Optimization of PPTGM and enzyme concentration

Three substrate concentrations (5 mg/mL, 10 mg/mL, and 15 mg/mL) and two crude enzyme concentrations (50 mg/mL and 100 mg/mL) as final concentrations were tested, in order to determine the appropriate substrate concentration for decreasing the reactor volume and enzyme concentration to reduce production costs. The

| Product name          | Declared function | Origin          | Declared optimum reaction conditions | Manufacturer    | Ginosenoide F1 content (% area/area) |
|-----------------------|-------------------|-----------------|--------------------------------------|----------------|------------------------------------|
| Control (PPTGM)       | —                 | —               | —                                    | —              | 0.0                                |
| Novozym 960           | Inulinas          | A. niger        | pH 4.1, Temp. 45–50                 | Novozyme       | 3.4                                |
| Viscozyme L           | β-Glucanase, hemicellulase | A. aculeatus             | pH 4.0, Temp. 45–50                  | Novozyme       | 35.7                               |
| Pectinex AFPL-4       | Pectin lyase      | A. aculeatus    | pH 4.3, Temp. 45–50                  | Novozyme       | 11.0                               |
| Pectinex Ultra SP-L   | Polygalacturonase | A. aculeatus    | pH 4.6, Temp. 45–50                  | Novozyme       | 7.6                                |
| Fungamyl 800L         | α-Amylase         | A. aculeatus    | pH 5.8, Temp. 45–50                  | Novozyme       | 3.8                                |
| Cellulase KN          | Cellulase, naringinase | A. niger    | pH 5.0, Temp. 45–50                  | Kyowa Chemical | 57.9                               |

PPTGM, protopanaxatriol-type ginsenoside mixture
Fig. 1. TLC analyses of time course of ginsenosides bioconversion by Cellulase KN. (A) Transformation of ginsenoside Re. (B) Transformation of ginsenoside Rg1. Developing solvent: CHCl₃:CH₃OH:H₂O (65:35:10, v/v, lower phase). Lanes S represent ginsenoside standards (PPT-type ginsenosides). PPT, protopanaxatriol.

Fig. 2. Transformation pathways of ginsenosides Re and Rg1 by the commercial enzyme Cellulase KN.

Fig. 3. Effect of the concentration of the PPTGM and Cellulase KN for the production of ginsenoside F1. PPTGM, protopanaxatriol-type ginsenoside mixture.
time course of the PPTGM and product F1 was determined via HPLC analyses in six test conditions (Fig. 3). In the test conditions of a low substrate concentration (5 mg/mL) and a high crude enzyme concentration (100 mg/mL), the PPTGM was completely converted to ginsenoside F1 within 24 h; this reaction speed is 1.3 times faster than that of the 5 mg/mL PPTGM with 50 mg/mL crude enzyme concentration and 1.6 times faster than that of 10 mg/mL PPTGM with 100 mg/mL crude enzyme concentration. Under the other three reaction conditions, the conversion was not completed within 48 h. These three reaction conditions were thus excluded in the next step. Owing to the advantages of the smaller usage of enzymes and complete conversion of Re, the conditions of 10 mg/mL substrate concentration and 100 mg/mL crude enzyme concentration were adopted for the next scaled-up biotransformation step.

3.4. Scaled-up production of ginsenoside F1 with purification

The enzyme reaction was carried out using Cellulase KN with PPTGM as the substrate with a concentration of 10 mg/mL, and Cellulase KN was adjusted to 100 mg/mL as the final concentration in 5 L in order to produce F1. Ginsenosides Re and Rg1 in the PPTGM were gradually changed to ginsenoside F1 with time, and 98% of ginsenoside Rg1 and 92% of Re were converted to F1 within 48 hours. This ginsenoside bioconversion was illustrated well in the HPLC analysis, as shown in Fig. 4A–E. This means that 11.3 g among 12.3 g Re and 9.9 g among 10.2 g of Rg1 in 50 g of PPTGM can be changed to ginsenoside F1 to produce 17.1 g among 17.9 g, theoretically.

In order to obtain high-purity ginsenoside F1, the enzymes, salt, and free sugars from the reaction mixture of the 5 L reaction of PPTGM with Cellulase KN were removed. About half of ginsenoside F1 precipitated to form a solid, whereas half of the remaining quantity dissolved in the supernatant (TLC data not shown). Three liters of a 95% ethanol solution was used, twice, to dissolve the precipitated ginsenoside F1 thoroughly. Ginsenoside F1 in the supernatant was purified using a column chromatograph packed with ODS (4L of the 95% methanol eluent). Both the eluent derived from the precipitate and the supernatant fraction were evaporated in vacuo, yielding 26.9 g of crude ginsenoside F1. Its chromatographic purity was 60.8±1.1%, as determined by HPLC. After purified using a silica column, 13.0 g of high-purity ginsenoside F1

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**Fig. 4.** HPLC analysis of the transformation of the PPTGM by Cellulase KN. (A) Standard ginsenosides. (B) PPTGM as a substrate for production of F1. (C) PPTGM reacted with Cellulase KN after 4 hours. (D) PPTGM reacted with Cellulase KN after 12 hours. (E) PPTGM reacted with Cellulase KN after 48 hours. (F) High-purity ginsenoside F1 after column purification packed with silica. PPTGM, protopanaxatriol-type ginsenoside mixture.

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**Fig. 5.** Entire process of F1 production using Cellulase KN. ODS, octadecylsilane; PPTGM, protopanaxatriol-type ginsenoside mixture.
(91.5 ± 1.1%) was finally obtained from 50 g of the PPTGM (Fig. 4F). The whole process is illustrated in Fig. 5. The PPTGM was primarily composed of ginsenosides Re and Rg1, in which ginsenosides Rb1, Rb2, Rb3, Rd, and Rg3(S) were also present. Among these ginsenosides, the total molar amount of Re and Rg1 that could be biotransformed into F1 using Cellulase KN was 243 mmol, which corresponds to 22.5 g of 50 g. The residue (27.5 g) consisted of other types of ginsenosides and unknown impurities. The molar amount of the produced high-purity ginsenoside F1 (13.0 g) was 18.6 mmol. This indicates that the recovery ratio through the biotransformation process using ginsenosides Re and Rg1 of the PPTGM to F1 reached 72.7% during the entire bioprocess engineering.

4. Discussion

Although F1 has antiaging, anticancer, and antioxidant effects, the production of high-purity ginsenoside F1 is limited by economic factors related to availability. For example, 20 mg of 98% ginsenoside F1 costs US $163.2 in Nanjing Zelang Medical Technology Co., Ltd. Thus far, high price and the lack of a selective technology for mass production of ginsenoside F1 have hampered its commercial use. To achieve increased production of F1, a number of researchers have sought to biotransform major ginsenosides Re or Rg1 into larger quantities of F1 using microorganisms and enzymes in laboratory settings [16,17,21]. Ko et al. [17] converted ginsenoside Re to F1 by a food-grade naringinase from P. decumbens, whereas Kim et al. [21] used the recombinant β-glucosidase from Sanguibacter keddii. However, the aforementioned studies were tested on small scales only to confirm the bioconversion ability. Furthermore, in order to use ginsenosides generated by microorganisms safely in consumables such as food, it is desirable to use food-grade microorganisms (GRAS, generally recognized as safe). Here, we report for the first time that the commercial enzyme Cellulase KN can be used to transform up to 10 mg/mL of the PPTGM to 10 gram-scale F1 within 48 h. Cellulase KN, capable of converting ginsenosides Re and Rg1 into F1, is expected to facilitate mass production of F1 from PPT-type ginsenosides Re and Rg1 derived from P. quinquefolius (American ginseng) or P. ginseng Meyer (Korean ginseng).

5. Conclusion

In summary, we describe the screening of a commercial enzyme, Cellulase KN, for the biotransformation of the major ginsenosides Re and Rg1 into the pharmacologically active rare ginsenoside F1. Optimized reaction conditions were obtained, and used for 10 gram-scale F1 production. The bioconversion process took place in a 10 L jar fermenter in 100mm acetic acid buffer (pH 5.0) at 50°C for 48 h, with an initial substrate concentration of 10 mg/mL. In terms of the yield, 13.0 g of F1 with 91.5 ± 1.1% chromatographic purity was obtained via the biotransformation of 50 g of the PPTGM. This is the first report of a commercial enzyme that is capable of 10 gram-scale production of F1 through biotransformation of the PPTGM followed by the purification steps.

Conflicts of interest

All authors declare no conflicts of interest.

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