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Production of the Rare Ginsenoside Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3) by Enzymatic Conversion Combined with Acid Treatment and Evaluation of Its Anti-Cancer Activity

Bong-Kyu Song[1,2], Kyeng Min Kim[1,2], Kang-Duk Choi[1,2], and Wan-Taek Im[1,2*]

[1]*Department of Biotechnology, Hankyong National University, Anseong 17579, Republic of Korea*
[2]*Genomic Informatics Center, Graduate School of Future Convergence Technology, Hankyong National University, Anseong 17579, Republic of Korea*

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*Corresponding author
Phone: +82-31-6705335;
Fax: +82-31-6705339;
E-mail: wandra@hknu.ac.kr

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The ginsenoside Rh2 has strong anti-cancer, anti-inflammatory, and anti-diabetic effects. However, the application of ginsenoside Rh2 is restricted because of the small amounts found in Korean white and red ginsengs. To enhance the production of ginsenoside Rh2-MIX (comprising 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3 as a 10-g unit) with high specificity, yield, and purity, a new combination of enzymatic conversion using the commercial enzyme Viscozyme L followed by acid treatment was developed. Viscozyme L treatment at pH 5.0 and 50°C was used initially to transform the major ginsenosides Rb1, Rb2, Rc, and Rd into ginsenoside F2, followed by acid-heat treatment using citric acid 2% (w/v) at pH 2.0 and 121°C for 15 min. Scale-up production in a 10-L jar fermenter, using 60 g of the protopanaxadiol-type ginsenoside mixture from ginseng roots, produced 24 g of ginsenoside Rh2-MIX. Using 2 g of Rh2-MIX, 131 mg of 20(*S*)-Rh2, 58 mg of 20(*R*)-Rh2, 47 mg of Rk2, and 26 mg of Rh3 were obtained at over 98% chromatographic purity. Then, the anti-cancer effect of the four purified ginsenosides was investigated on B16F10, MDA-MB-231, and HuH-7 cell lines. As a result, these four rare ginsenosides markedly inhibited the growth of the cancer cell lines. These results suggested that rare ginsenoside Rh2-MIX could be exploited to prepare an anti-cancer supplement in the functional food and pharmaceutical industries.

Keywords: Biotransformation, ginsenoside, ginsenoside Rh2, Viscozyme L, mass production, acid-heat treatment

Introduction

Panax ginseng C.A. Meyer has long been used as an herbal medicine in East Asian countries such as Korea, China, Japan, and Vietnam [1]. The major pharmacologically active compounds in ginseng are called ginsenosides, which show various activities, including anti-cancer, antitumor, anti-fatigue, anti-inflammatory, and anti-diabetic effects [2]. The minor ginsenosides, including F1, F2, Rg3, Rh1, compound Y, compound Mc, and compound K, can be produced by hydrolysis of the sugar moieties of the major ginsenosides (Rb1, Rb2, Rc, Rd, Re, and Rg1) [3]. Various methods can be used to transform the minor ginsenosides from the major ginsenosides, such as acid treatment, heating [4], microbial treatment, and enzymatic transformation [5]. For example, ginsenoside F1 has been produced via the bioconversion of ginsenoside Rg1 using Cellulase KN [6]. In addition, the major ginsenosides Rb2 and Rc were converted into Rg3 or Rh2 by enzyme treatment [7,8]. Likewise, Rh1, Rg3, and Rh2 were produced from raw ginsenosides using 0.01% formic acid and heating at 120°C for 4 h [9].

The minor ginsenoside Rh2 is one of the bioactive components that can be transformed from a major ginsenoside. Rh2 can inhibit the growth of many kinds of cancer cells, including breast cancer, prostate cancer,

hepatoma, gastric cancer, colon carcinoma, and pancreatic cancer [10]. For example, the ginsenoside 20(*S*)-Rh2 has anti-cancer effects in the human acute leukemia cell line Reh via a mechanism involving mitochondrial cytochrome *c* and activation of caspase-9 and caspase-3 [11]. Rh2 was also shown to induce apoptosis of the human hepatoma cell line SK-HEP-1 by the activation of caspase-8 and caspase-9 [12], and to inhibit migration of HepG2 cells by inhibiting the expression of MMP3 at the mRNA and protein levels [13]. The ginsenoside Rh2 can inhibit the growth of hepatocellular carcinoma cells significantly through coordinated autophagy and β-catenin signaling [14]. The cytotoxic effect of ginsenosides Rk2 and Rh3, which are the dehydroxylated structures of Rh2 at the C20 position of the aglycone, extracted from steamed ginseng leaves, on HL-60 cells has been reported [15]. Furthermore, ginsenoside Rh2 has anti-inflammatory effects via the inhibition of lipopolysaccharide (LPS)-induced activation of microglia and overproduction of inflammatory mediators via modulation of the TGF-beta1/Smad Pathway [16]. In addition, Rh2 has an anti-obesity effect related to the activation of the adenosine monophosphate-activated protein kinase signaling pathway in 3T3-L1 adipocytes [17].

Ginsenoside Rh2 can be produced using microbes or enzymatic processes. For example, *Esteya vermicola* CNU 120806 has β-glucosidase activity and can convert ginsenoside Rg3 into Rh2 [18]. Human intestinal bacteria could also perform the transformation [19]; however, the authors only

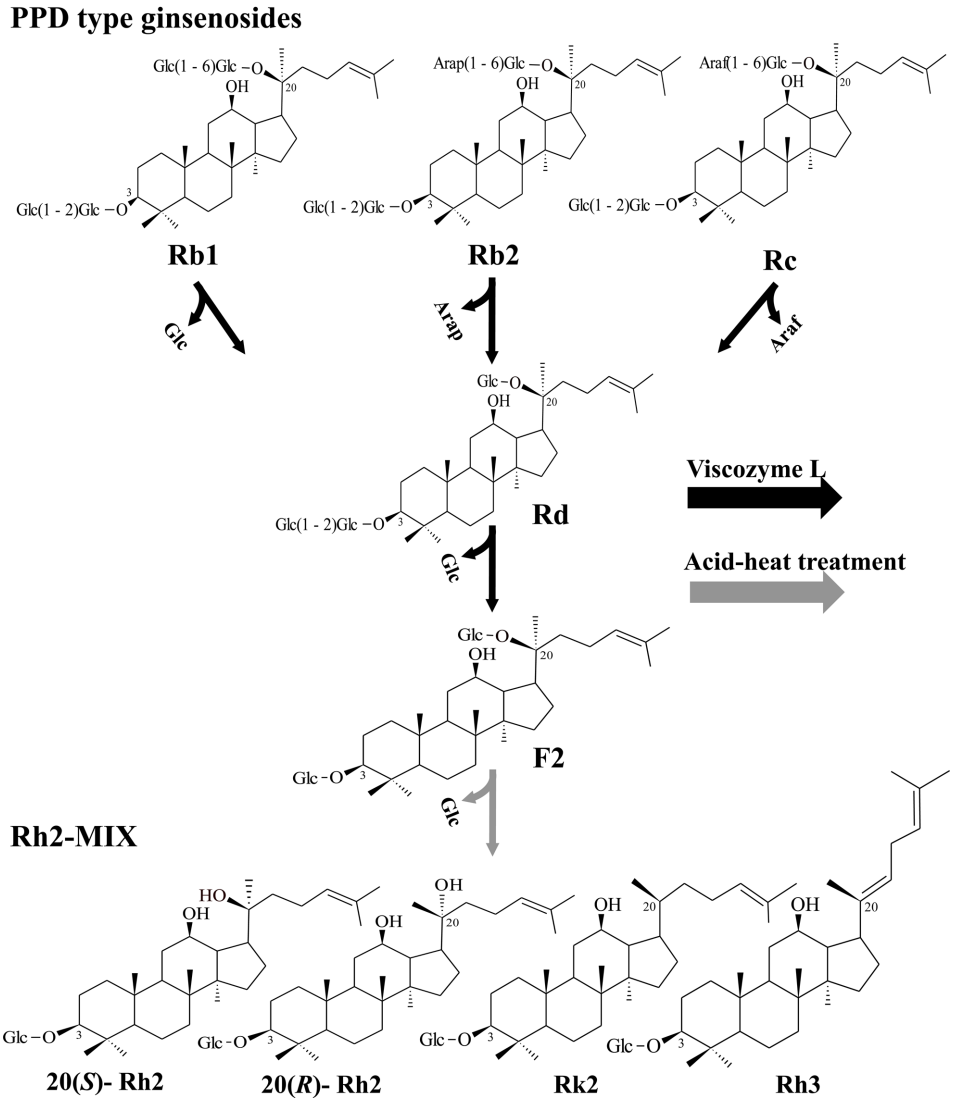

Fig. 1. Transformation pathway for the production of ginsenoside Rh2-MIX from a protopanaxadiol-type ginsenoside mixture by Viscozyme L treatment followed by acid-heat treatment using citric acid.

presented a simple enzymatic digestion experiment, without further scale-up or process engineering. To date, several recombinant ginsenoside hydrolyzing enzymes have been constructed to produce ginsenosides 20(*S*)-Rg3 [20], F2 [21], 20(*S*)-Rg2 [22], 20(*S*)-Rh1 [23], and F1 [6] on a large scale However, there is no report of the production of Rh2 using bioprocess engineering.

In this study, an effective transformation method was developed to obtain the rare ginsenoside Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3) from protopanaxadiol (PPD)-type ginsenosides. To achieve this objective, a commercial food-grade enzyme (Viscozyme L) that can optimally transform the major ginsenosides Rb1, Rb2, Rc, and Rd into F2 was screened, and acid-heat treatment was then used to convert F2 to Rh2-MIX by cleaving the inner glucose moiety at the C20 position (Fig. 1). Subsequently, the rare ginsenosides 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3 were purified by recycling preparative high-performance liquid chromatography (RP-HPLC). In addition, the cytotoxic effects of the rare ginsenosides 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3 were tested on human cellular carcinoma HuH-7, breast cancer MDA-MB-231, and murine melanoma B16F10 cell lines.

Materials and Methods

Materials

Ginsenoside standards at over 98% purity, such as Rb1, Rb2 Rc, Rd, 20(*S*)-Rh2, 20(*R*)-Rh2, F2, Rk2, and Rh3, were purchased from Nanjing Zelang Medical Technology Co. Ltd. (China). The PPD-type ginsenoside mixture (PPDGM) was extracted from the dried roots of 2 kg of Korean ginseng (*Panax ginseng* C. A. Meyer) together with 2 kg of *Panax quinquefolius*. Four kilograms of ginseng root powder was extracted twice with 40 L of 70% ethanol. 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 (400 mm long × 100 mm deep) packed with Diaion HP-20 resin (Mitsubishi Chemical, Japan). Free sugar molecules and unwanted hydrophilic compounds from the HP-20 absorbed beads were washed with eight column volumes of water, and followed by eight column volumes of 36% ethanol. Finally, PPD-type ginsenosides were eluted using six column volumes of 80% ethanol. The ethanol extracts were evaporated in vacuo, and the dried residue was used as the substrate ginsenoside for Rh2-MIX production. According to the HPLC analysis, the PPDGM comprised mainly Rb1 (328 mg/g), Rc (173 mg/g), Rb2 (98 mg/g), Rb3 (25 mg/g), Rd (107 mg/g), Rg3 (12 mg/g), and small amounts of other ginsenosides. The PPDGM was dissolved in 50 mM of acetate buffer, which could solubilize PPDGM at up to 100 mg/ml. HPLC-grade methanol and acetonitrile were obtained from SK Chemical Co., Ltd. (Korea). Citric acid with a purity of 99% was purchased from Samchun Chemical Co., Ltd. (Korea). The other chemicals used in this study were of analytical grade or higher.

Screening of Commercial Enzymes to Transform PPD-Type Ginsenosides

The PPDGM was diluted in 500 µl of acetate buffer (pH 5.0) as a 1% (w/v) solution, and the same volumes of different commercial enzymes (Viscozyme L (Novozyme, Denmark), Pectinex Ultra AFP (Novozyme), Fungamyl 800L (Novozyme), Lactazyme A (Genofocus, Korea), Dextrozyme DX 2X (Novozyme), Ultraflo L (Novozyme), and Novarom Blanc (Novozyme) were added for subsequent testing. The reaction mixture was placed on a shaking incubator for 48 h at 200 rpm. The reactants were sampled at regular intervals and analyzed via thin-layer chromatography (TLC) or HPLC after pretreatment (see Analytic Methods section).

Transformation of the PPD-Type Ginsenosides Using Viscozyme L Followed by Acid-Heat Treatment

The transformation activity of combined treatment of Viscozyme L followed by acid-heat treatment was examined to determine the specificity and selectivity of the transformation of PPD-type ginsenosides Rb1, Rc, Rb2, and Rd. The transformation involved hydrolysis of the glucose or arabinose moieties attached at the C3 and C20 sites of the PPD-type ginsenosides. First, the enzyme solution (100 mg/ml in 100 mM acetate buffer, pH 5.0) was reacted with an equal volume of the PPD-type ginsenosides at a concentration of 10 mg/ml in 100 mM acetate buffer (pH 5.0) at 50°C for 48 h. Second, the enzyme-treated reactants were heat-treated at 120°C for 15 min with 2% (w/v) citric acid. Samples were taken at regular intervals and analyzed via TLC or HPLC after pretreatment (see Analytic Methods).

Scaled-Up Transformation of PPDGM to Ginsenoside Rh2-MIX

The transformation was performed in a 10-L stirred-tank reactor (Biotron GX; Hanil Science Co. Ltd, Korea) with a 6 L working volume and stirring at 200 rpm. The reaction was performed under pH 5.0 at 50°C for 48 h. The reaction comprised 10 mg/ml of substrate ginsenoside (PPDGM; total 60 g) and 1.5 L of Viscozyme L. After confirmation that the PPDGM had been almost completely biotransformed to ginsenoside F2, the reaction mixture was heat-treated at 120°C for 15 min with 2% (w/v) citric acid. Samples were collected at regular intervals and analyzed by HPLC to determine the transformation of the PPDGM to ginsenoside Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3).

Purification and Separation of Ginsenoside Rh2-MIX as Single Components

Following the 6 L reaction of PPDGM using Viscozyme L followed by acid-heat treatment with citric acid, the mixture was cooled to 4°C and centrifuged at 12,000 rpm for 15 min (Component R; Hanil Science Co. Ltd.). The transformed ginsenosides, including 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3, in precipitate were processed

to remove the enzymes, salt, and free sugars from the reaction mixture. The precipitate was dissolved in 6.0 L of 95% ethanol solution and then evaporated in vacuo. The crude ginsenoside Rh2-MIX was purified using a Biotage SNAP flash chromatography cartridge (180 × 70 φ mm; Biotage, Sweden) packed with 340 g of silica resin (230–400 mesh). The cartridge was equilibrated with chloroform, and 24 g of powdered crude ginsenoside Rh2-MIX was dry-loaded into a self-packed sample cartridge. Elution was performed with three bed volumes of chloroform and five bed volumes of chloroform-methanol-water (89.5:10.5:1 (v/v)). Fractions were taken for every 340-ml elution (0.5 bed volume). The results were analyzed by HPLC. One gram each of the ginsenoside metabolites in fraction 1 and fraction 2 were purified using a recycling preparative HPLC system (LC-9201; Japan Analytical Instruments) equipped with a UV/refractive index detector and a reverse-phase column (octyldecylsilane (ODS), 500 × 20 mm; inside diameter (i.d.), 15 μm). An isocratic solvent system of CH_3CN and deionized H_2O (95:5) was used, and the detection wavelength was set at 203 nm. The resulting powder was dissolved in 100% methanol and analyzed by HPLC.

Cell Culture

The murine melanoma cell line B16F10 and the human breast cancer cell line MDA-MB-231 were obtained from the ATCC (USA). The human hepatocellular carcinoma cell line HuH-7 was obtained from the JCRB (Japan). All the cell lines were cultured in Dulbecco's modified Eagle medium (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (HyClone) at 37°C in a humidified incubator with 5% CO_2.

Cell Viability Assay and Anti-Cancer Effect

To evaluate the anti-cancer effect of ginsenosides 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, Rh3, and Rh2-MIX on cell viability, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) assay was used for the B16F10, MDA-MB-231, and HuH-7 cell lines. Cells of B16F10, HuH-7, and MAD-MB-231 were seeded at 2×10^3, 7×10^3, and 5×10^3 cells per well, respectively, in 96-well microtiter culture plates and incubated for 24 h. The cancer cells were then treated with five concentrations (0, 12.5, 25, 50, and 100 μM) of the ginsenosides for 24 h. Controls were exposed to culture medium with dimethyl sulfoxide (DMSO) vehicle. After 24 h, the cells were treated with 500 μg/ml of MTT solution, and incubated at 37°C with 5% CO_2 for 4 h. The medium was then removed and 100 μl of DMSO was added to dissolve the blue formazan crystals converted from MTT. Absorbance at 540 nm was measured using a microplate reader (SpectraMax Plus 384; Molecular Devices, USA).

Analytic Methods

TLC analysis. TLC was performed using $60F_{254}$ silica gel plates (Merck, Germany) with $CHCl_3$-CH_3OH-H_2O (65:35:10, lower phase) as the solvent. The spots on the TLC plates were identified by comparison with those of standard ginsenosides after visualization by spraying with 10% (v/v) H_2SO_4, followed by heating at 110°C for 5 min.

HPLC analysis. HPLC analysis of the ginsenosides was performed using an HPLC system (Younglin Co., Ltd., Korea), with a quaternary pump, an automatic injector, and a single-wavelength UV detector (model 730D), and the results were analyzed using Younglin's AutoChro 3000 software for peak identification and integration. The separation was carried out on a Prodigy ODS(2) C_{18} column (5 μm, 150 × 4.6-mm i.d.; Phenomenex, USA) with a guard column (5 μm, 12.5 × 4.6 mm i.d.; Eclipse XDB C_{18}). The mobile phases were A (acetonitrile) and B (water). The gradient elution started with 32% solvent A and 68% solvent B, and was changed to the following: from 0–8 min, A was increased from 32% to 65%; from 8–12 min, A was increased from 65% to

Table 1. Relative ginsenoside F2 contents produced from a protopanaxadiol-type ginsenoside mixture (PPDGM) reacted with several commercial enzymes.

Product name	Declared function	Origin	Declared optimum reaction conditions		Manufacturer	Ginsenoside F2 content (%, area/area)
			pH	Temp.		
Control (PPDGM)	-	-	-	-	-	0.0
Viscozyme L	β-Glucanase, hemicellulase	*Aspergillus aculeatus*	4.0	45–50	Novozyme	83.6
Pectinex Ultra AFP	Pectin lyase	*Aspergillus aculeatus* *Aspergillus niger*	4.3	45–50	Novozyme	46.7
Novarom Blanc	Polygalacturonase	*Aspergillus niger*	4.5	45–50	Novozyme	42.9
Fungamyl 800L	α-Amylase	*Aspergillus oryzae*	5.8	45–50	Novozyme	10.3
Lactazyme A	Exo-(1 → 4)-β-D-galactosidase	*Aspergillus niger*	5.0	45–50	Genofocus	1.2
Dextrozyme DX 2X	Gluco-amylase, pullulanase	*Aspergillus niger* *Bacillus subtilis*	4.5	45–50	Novozyme	18.3
Ultraflo L	β-Glucanase	*Humicola insolens*	4.5	45–50	Novozyme	7.4

100%; from 12–15 min, A was constant at 100%; from 15–15.1 min, A was decreased from 100% to 32%; from 15.1–25 min, A was constant at 32%. The flow rate was 1.0 ml/min, and detection was performed by monitoring absorbance at 203 nm and with an injected volume of 25 μl.

Results

Selection of the Most Effective Enzyme for the Transformation of Ginsenoside F2

We examined the transformation of PPDGM into ginsenoside F2 using seven types of commercial enzymes for 2 days. The ginsenoside F2 contents transformed from the PPDGM by the enzymes were determined as the percentage of ginsenoside F2 among the entire measureable ginsenoside peak area using HPLC analysis. As shown in Table 1, the commercial enzymes Pectinex Ultra AFP, Fungamyl 800L, Lactazyme, Dextrozyme DX 2X, Ultraflo L, and Novarom Blanc could not transform all of the ginsenosides Rb1, Rb2, Rc, and Rd to F2. However, Viscozyme L effectively transformed ginsenosides Rb1, Rc, Rb2, and Rd into F2. The results showed that among the seven enzymes tested, the food-grade enzyme Viscozyme L was the most effective to transform the PPDGM (Rb1, Rb2, Rc, and Rd) to F2. Therefore, Viscozyme L was chosen for further study.

Transformation Activity of the PPD-Type Ginsenosides Using Viscozyme L Followed by Acid-Heat Treatment

For verification of the transformation pathway of the four PPD-type ginsenosides (Rb1, Rc, Rb2, Rd) through the combinative treatment of Viscozyme L followed by acid-heat treatment with citric acid, HPLC analyses were performed at regular intervals. It is clear that the Viscozyme L could transform four PPD-type ginsenosides (Rb1, Rc, Rb2, and Rd) into ginsenoside F2 for 48 h, based on the peak retention time (Figs. 2A–2C). No further reaction occurred after a long time reaction. After adding the 2% (w/v) citric acid to the enzyme-treated reactant by heat-treatment at 120°C for 15 min, ginsenoside F2 was fully transformed to Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2 and Rh3) (Fig. 2D). Thus, it is concluded that Viscozyme L efficiently transformed ginsenosides Rb1, Rc, Rb2, and Rd to ginsenoside F2 by hydrolyzing the outer glucose moiety at the C3 position and the outer arabinose or glucose moieties at the C20 position. Then, acid-heat treatment using citric acid as the pH modulator finally transformed ginsenoside F2 into Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3) by cleaving the inner glucose moiety at C20 position. This acid-heat

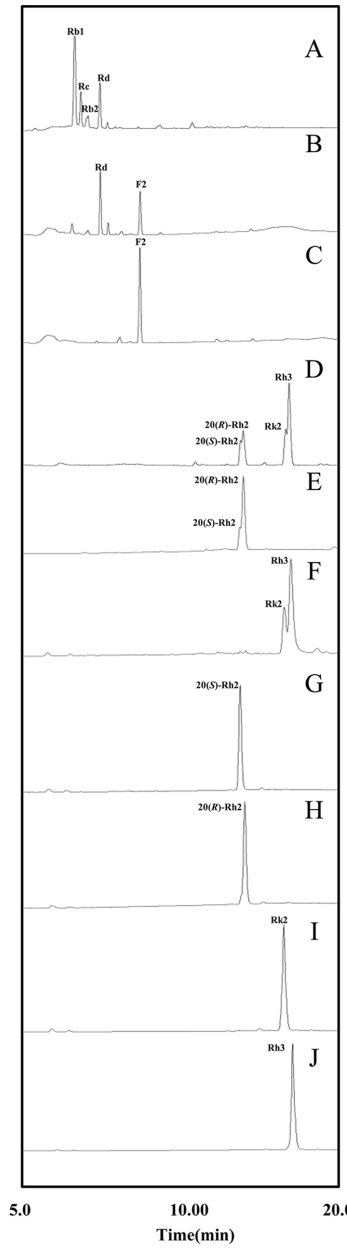

Fig. 2. High-performance liquid chromatography chromatogram of the transformation of the protopanaxadiol-type ginsenoside mixture (PPDGM) by Viscozyme L treatment followed by acid-heat treatment using citric acid, and the purification of rare ginsenosides 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3.
(A) Substrate, PPDGM; (B) the reaction mixture after Viscozyme L treatment for 24 h; (C) the reaction mixture after Viscozyme L treatment after 48 h; (D) the reaction mixture after acid-heat treatment at 121°C for 15 min using citric acid; (E) fraction 1 after column purification; (F) fraction 2 after column purification; (G) ginsenoside 20(*S*)-Rh2 after isolation using recycling preparative HPLC; (H) isolated ginsenoside 20(*R*)-Rh2; (I) isolated ginsenoside Rk2; and (J) isolated ginsenoside Rh3.

treatment process cleaving the inner glucose moiety at the C20 position is a well-known process to make Rg3-MIX (20(*S*)-Rg3, 20(*R*)-Rg3, Rk1, and Rg5) from major PPD-type ginsenosides [8]. The above results indicated that the combinative process of enzymatic method followed by acid-heat treatment is highly effective for production of Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3) from ginsenosides Rb1, Rc, Rb2, and Rd. Therefore, the proposed transformation pathways are as follows: (Rb1, Rb2, Rc) → Rd → F2 → Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3) (Fig. 1).

Scaled-Up Production of Ginsenoside Rh2-MIX with Purification

The enzyme reaction was started using Viscozyme L with PPDGM as the substrate with a concentration of 10 mg/ml in 6 L in order to produce Rh2-MIX. The ginsenosides Rb1, Rb2, and Rc were gradually transformed to ginsenoside F2 via Rd with time and 99% of four kinds of major ginsenosides were transformed to F2 within 48 h. Then, the mixture was heated at 121°C for 15 min adding 2% (v/w) citric acid (120 g), and ginsenoside F2 was ultimately transformed to ginsenoside Rh2-MIX. From the chromatographic images of reaction samples drawn from different time points and analyzed via HPLC, it was demonstrated that the biotransformation rate was nearly complete for ginsenosides Rb1, Rb2, Rc, Rd, and F2, which were no longer detected by the HPLC analysis. After the purification step removing enzymes, salt, and free sugars from the reaction mixture of the 6 L reaction, 24 g of crude Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3) was obtained.

Purification of Rare Ginsenosides 20(S)-Rh2, 20(R)-Rh2, Rk2, and Rh3

To purify the rare ginsenosides 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3 individually, 24 g of powdered crude ginsenoside Rh2-MIX was dry-loaded into a self-packed silica cartridge. After gradient chloroform-methanol-water elution (89.5:10.5:1), 5.2 g of fraction 1 (20(*S*)-Rh2 and 20(*R*)-Rh2) (Fig. 2E) and 7.0 g of fraction 2 (Rk2 and Rh3) (Fig. 2F) were finally obtained. Fraction 1 (1 g) and fraction 2 (1 g) were subjected to recycling preparative HPLC, which provided 131 mg of 20(*S*)-Rh2, 58 mg of 20(*R*)-Rh2, 47 mg of Rk2, and 26 mg of Rh3, respectively (Fig. 2G-J). The chromatographic purity of the four ginsenosides was more than 98%, as determined by HPLC. The whole process is illustrated in Fig. 3.

Anti-Cancer Effect of Ginsenosides 20(S)-Rh2, 20(R)-Rh2, Rk2, and Rh3 and Rh2-MIX

Treatment with the ginsenosides 20(*S*)-Rh2, Rk2, Rh3, and Rh2-MIX caused significant cell death among B16F10, MDA-MB-231, and HuH-7 cancer cells at the tested concentrations. The IC_{50} (half maximal inhibitory concentration) values of 20(*S*)-Rh2, Rk2, Rh3, and Rh2-MIX on B16F10 cancer cells were 35.6, 77.8, 79.3 and 44.0 μM; on MDA-MB-231 cancer cells were 39.9, 56.2, 56.3 and 57.9 μM; and on HuH-7 cancer cells were 39.0, 40.8, 16.7 and 38.7 μM, respectively (Fig. 4). These data suggested that ginsenosides 20(*S*)-Rh2, Rk2, Rh3, and Rh2-MIX possess anti-cancer effect on the B16F10, MDA-MB-231, and HuH-7 cancer cell lines. This anti-cancer effect was very similar to that disclosed in a previous report [9–14]. By contrast, 20(*R*)-

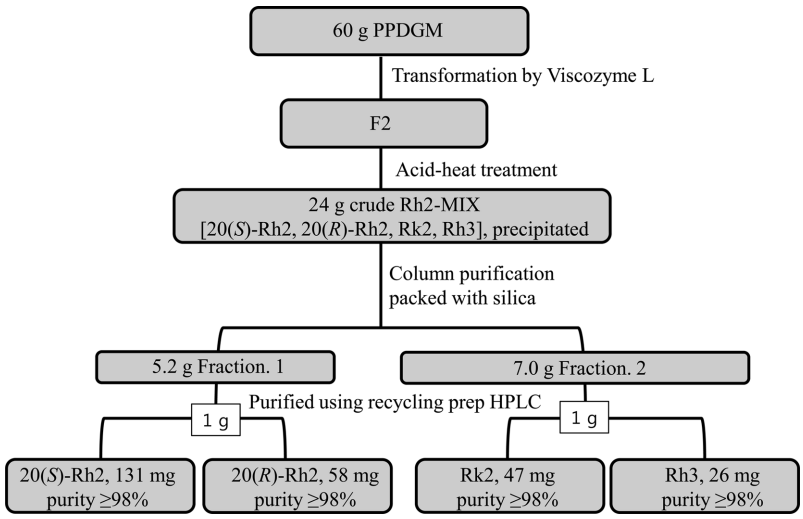

Fig. 3. The whole process of production of Rh2-MIX from a protopanaxadiol-type ginsenoside mixture (PPDGM) and the purification of rare ginsenosides 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3.

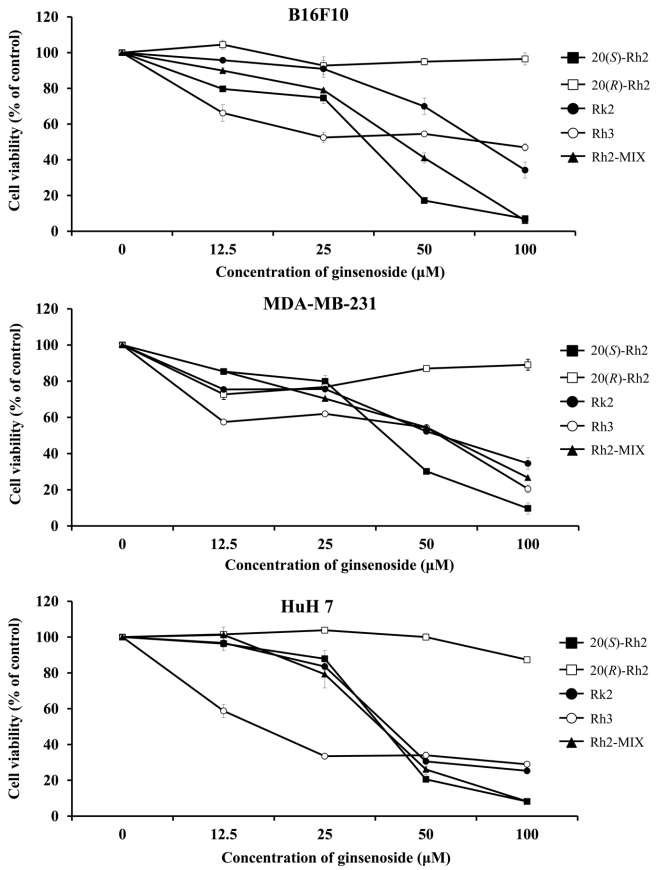

Fig. 4. Effects of 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3 on cell proliferation of B16F10, MDA-MB-231, and HuH-7 cells.
Cells were treated with 12.5–100 μM 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, Rh3, and Rh2-MIX for 24 h. Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Rh2 did not inhibit B16F10, MDA-MB-231, and HuH-7 cancer cell growth at various concentration ranges within 24 h (Fig. 4), which suggested that 20(*R*)-Rh2 has no anti-cancer efficacy. This agreed with previous reports in which the cytotoxicity of 20(*R*)-Rh2 to the prostate cancer cell line LNCaP or pre-osteoclastic cell line RAW 264 was studied [24, 25].

Discussion

The rare ginsenosides 20(*S*)-Rh2, Rk2, and Rh3 have remarkable anti-cancer effects on various types of cancers [9, 15], similar to that of ginsenoside C-K. In particular, the efficacy of 20(*S*)-Rh2, including anti-cancer, anti-hyperglycemic, and anti-obesity effects [26], has been reported in many previous publications. However, the production of high-purity ginsenoside Rh2 or its analogs (Rk2, Rh3) has been limited by a lack of ginsenoside transformation technology. The high price and lack of mass production of ginsenoside Rh2 have hampered its commercial use.

To date, many studies have attempted to find suitable enzymes to develop a bioprocess to transform major ginsenosides to minor ginsenoside, such as for F2 [21], 20(*S*)-Rg3 [20], C-K [27], Rh1 [23], 20(*S*)-Rg2 [22], and F1 [6]. For example, β-glucosidase cloned from *Microbacterium esteraromaticum* could effectively transform ginsenosides Rb1 and Rd into 20(*S*)-Rg3 by hydrolysis of the outer and inner glucoses at the C20 position [23]. However, there is no report of the production of ginsenoside Rh2 by transformation from major ginsenosides directly because of the difficulty of cleaving the three glucose moieties specifically (outer and inner glucoses at the C3 position, and the outer glucose at the C20 position). By contrast, there are many reports of biotransformation to produce ginsenoside C-K, which has a very similar structure and function to Rh2. A Korean cosmetic company succeeded in producing C-K in large quantities using a commercial enzyme (a kind of pectinase) and used C-K as the main active component in the brand "Sulwha Soo." However, a large-scale production process for ginsenoside Rh2 had not been developed. Therefore, we attempted to develop an efficient method to make ginsenoside Rh2 using a combined biotransformation and acid-heat treatment. This combined method was necessary because there is no effective enzyme that can transform major ginsenosides to ginsenoside Rh2 directly.

There are two ways to make ginsenoside Rh2. One is cleavage of the inner glucose moiety at the C20 position of the aglycone of ginsenoside F2, and the other is to cleave the outer glucose moiety at the C3 position of aglycone of Rg3. We selected the first method to make food-grade ginsenoside Rh2 because there is no reported food-grade commercial enzyme that can cleave the outer glucose moiety specifically at the C3 position of the aglycone. Unfortunately, acid-heat treatment is inevitable to produce a racemic mixture of Rh2 (20(*S*)-Rh2 and 20(*R*)-Rh2) and its analogs, Rk2 and Rh3, which are dehydroxylated to Rh2 at the C20 position of the aglycone from ginsenoside F2.

Here, we report the enhanced production of the rare ginsenoside Rh2-MIX (20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3) using a food-grade commercial enzyme, Viscozyme L, for the biotransformation of the major ginsenosides to F2, followed by citric acid-heat treatment, producing 10 g units of Rh2-MIX using a 10 L jar fermenter. In terms of yield, 24.0 g of Rh2-MIX at 74.3 ± 1.5% (the sum of the area of

20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3) chromatographic purity was obtained from 60 g of PPDGM, which opened up the possibility of the commercial use of Rh2-MIX at low cost.

Finally, we tested the anti-cancer efficacy of ginsenoside Rh2-MIX in comparison with the single ginsenosides 20(*S*)-Rh2, 20(*R*)-Rh2, Rk2, and Rh3, because the method to prepare Rh2-MIX is much simpler than that used to prepare the single ginsenosides. Rh2-MIX showed good anti-cancer efficacy against B16F10, MDA-MB-231, and HuH-7 cancer cells, similar to that of the single ginsenoside 20(*S*)-Rh2, indicating that Rh2-MIX could substitute for the single ginsenoside compound 20(S)-Rh2 as an anti-cancer health supplement.

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