Korean Ginseng Berry Fermented by Mycotoxin Non-producing 
*Aspergillus niger* and *Aspergillus oryzae*: Ginsenoside Analyses and Anti-proliferative Activities

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To transform ginsenosides, Korean ginseng berry (KGB) was fermented by mycotoxin non-producing *Aspergillus niger* and *Aspergillus oryzae*. Changes of ginsenoside profile and anti-proliferative activities were observed. Results showed that *A. niger* tended to efficiently transform protopanaxadiol (PPD) type ginsenosides such as Rb1, Rb2, Rd to compound K while *A. oryzae* tended to efficiently transform protopanaxatriol (PPT) type ginsenoside Re to Rh1 via Rg1. Butanol extracts of fermented KGB showed high cytotoxicity on human adenocarcinoma HT-29 cell line and hepatocellular carcinoma HepG2 cell line while that of unfermented KGB showed little. The minimum effective concentration of *niger*-fermented KGB was less than 2.5 μg/mL while that of *oryzae*-fermented KGB was about 5 μg/mL. As *A. niger* is more inclined to transform PPD type ginsenosides, *niger*-fermented KGB showed stronger anti-proliferative activity than *oryzae*-fermented KGB.

Key words Korean ginseng berry (KGB); ginsenoside; transformation; mold; anti-proliferation; mycotoxin

Ginseng (the root of *Panax ginseng* C. A. MEYER, family Araliaceae) has been used as a kind of precious remedy for thousands of years. Until the 1960s, it has been a mystery as to why ginseng had such remarkable results. Consequently, researchers discovered that ginsenosides were the main components responsible for its biological activities (Table 1). These ginsenosides show various activities including anti-diabetic1,2) and anti-tumor effects.3,4) In addition, the berry has a ginsenoside profile distinct from the root.5) Korean ginseng berry (KGB) has a propanaxatriol (PPT) type ginsenoside as its main ginsenoside while the root has the protopanaxadiol (PPD) type. KGB is particularly known for its high ginsenoside Re content, which is about 30 times higher than what is present in the root.5) Moreover, KGB is also different with American ginseng berry (AGB), which contains ginsenoside Rh3, a PPD type, as its main ginsenoside.6)

After ingesting herbal medicine, the main active ingredients will be transformed to their nonpolar forms by the intestinal microflora before absorption in the gastrointestinal tract.7,8) Tawab et al.9) reported the ginsenoside compound K (cK), Rh1 and F1, rather than the underdeglycosylated forms that were detected in human plasma and urine after ginseng powder was administrated to volunteers. Therefore, the biological activities of ginsenosides may largely depend on the metabolic activity of intestinal microflora. Since the intestinal microflora vary among individuals, about 20% of people cannot efficiently, or even at all, transform ginsenosides.10) This may explain why some people using ginseng achieved their expectations while others did not. Moreover, deglycosylated ginsenosides show more efficient activity than the underdeglycosylated forms.11) Consequently, it is necessary to transform ginsenosides before ingesting purely ginseng.

Various deglycosylation methods like mild acid hydrolysis12) alkaline cleavage13) and enzymatic hydrolysis14) are limited because they are hard to use in food and pharmaceuticals, which have led to microbial conversion. However, microorganisms used to transform ginsenosides are often not of a food-grade standard. In previous studies,15,16) we have researched the transformation of ginsenosides by various microorganisms that have been safely used for foods consumption. Some kinds of mold also have strong deglycosylating power and have long been used to ferment traditional foods in China, Korea and other countries. In the present study, we utilized the mycotox-

### Table 1. Chemical Structures of Ginsenosides

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<td>R1</td>
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in non-producing *Aspergillus (A.) niger* and *Aspergillus (A.) oryzae* to transform ginsenosides in KGB and observed the changes in the ginsenoside profile and the anti-proliferative activity.

**MATERIALS AND METHODS**

**Materials** Korean ginseng berry (the berry of *Panax ginseng* C.A. Meyer, family Araliaceae) was provided by Korean Genetic Pharm (Seoul, Korea). Standard ginsenosides such as Rb1, Rb2, Rd, Rg1, and F1 were purchased from Biotech (Nanjing, China). Ginsenoside Re, Rg2, Rg3, F2, Rh1, Rh2, and cK were purchased from Cogn Biotech (Chengdu, China). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Fetal bovine serum (FBS), trypsin–ethylenediaminetetraacetic acid (EDTA), and antibiotic–antimycotic (AA) solution were Gibco products purchased from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.). Other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise mentioned.

**Fermentation of KGB** Dried KGB was powdered and mixed with distilled water in 1:10 ratio before being extracted at 80°C for 3 h in a water bath with shaking. The extract was filtered with 4 layers of gauze and the filtrate used as culture medium. *A. niger* KACC 46494 and *A. oryzae KACC* 40247 were purchased from Korean Agricultural Culture Collection (Jeonju, Korea) and were cultured with potato dextrose agar (PDA) medium under aerobic conditions at 30°C for several days. The spores were scraped from PDA plate and suspended in saline containing 0.005% Tween 80. The collected spores were inoculated at a density of 10^6 spores/mL in the prepared medium. The culture broth was incubated at 30°C under aerobic conditions with shaking.

**Analysis of Mycotoxins** Aflatoxin, ochratoxin, cyclopiazonic acid, and fumonisin were analyzed by HPLC methods after fermentation according to previous studies.17,18

**Preparation of Fermented KGB Samples** Fermented culture broth was freeze-dried and extracted with n-butanol at 80°C for 1 h. After filtration with Whatman No. 41 filter paper (Kent, U.K.), the filtrate was evaporated using a speed vacuum concentrator. The butanol extracts were used as samples for TLC analysis, HPLC analysis, and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

**Analysis of Ginsenosides Using TLC** Silica gel 60 F254 plate of Merck KGAa (Darmstadt, Germany) was used to conduct TLC analysis. A mixture of chloroform–methanol–water (65:35:10, v/v/v) was stirred overnight before the lower phase was used as the mobile phase. The plate was stained by spraying with H2SO4 in ethanol (10:90, v/v) and followed by heating.

**Analysis of Ginsenosides Using HPLC** The butanol extract of fermentation broth was analyzed by HPLC conducted with a Agilent HP 1090 Series instrument (Santa Clara, CA, U.S.A.) and a diode array detector (DAD). A Poroshell 120 EC-C18 column (3×100 mm, 2.7 μm) from Agilent was used. The mobile phase consisted of water (A) and acetonitrile (B). The elution condition was optimized as follows: 0–16 min (19% B), 16–21 min (19–27% B), 21–30 min (27–29% B), 30–47 min (29–40% B), and 47–65 min (40–80% B). As for analysis of ginsenoside Rg2 and Rh1, an adjusted elution condition was used, which was 0–16 min (19% B), 16–35 min (19–30% B). The flow rate, injection volume, and detection wavelength were set as 0.5 mL/min, 20 μL, and 203 nm, respectively. Standard curves were constructed from the measured peak areas and the related concentration of ginsenosides. Various kinds of ginsenosides in the extract were identified by comparison of their retention times with those of ginsenoside standards. The contents of ginsenosides in each sample were calculated using standard curves.

**Cell Culture** A human colorectal adenocarcinoma HT-29 (KCLB 30038) cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). A human liver hepatocellular carcinoma HepG2 (HB-8065) cell line was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Both cell lines were subcultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) FBS and 1% (v/v) AA solution at 37°C in a humidified atmosphere of 95% air and 5% CO2.

**Cell Proliferation Assay** The butanol extracts of KGB were dissolved in dimethyl sulfoxide (DMSO) and were stored at −20°C before use. Cells were seeded at a density of 2×10^5 cells per well in a 96-well plate and allowed to adhere for 24 h. Various concentrations of extracts were added to the well and the final concentration of DMSO was adjusted to 0.5%. After 48 h of exposure, the supernatant was discarded and 30 μL of MTT solution (1 mg/mL) was added to each well and
followed by incubation at 37°C for 2 h. Finally, the supernatant was removed and 150 µL of DMSO was added to solubilize the formed formazan salt with shaking. The relative amount of formazan salt was determined by the absorbance at 490 nm using a microplate reader (Philadelphia, PA, U.S.A.).

**Statistical Analysis** Results are presented as the mean±standard error (S.E.). ANOVA followed by Duncan multiple comparison was utilized to test whether the results had statistical significance or not. The level of statistical significance was set at *p*<0.05.

**RESULTS AND DISCUSSION**

**Detection of Mycotoxins in Fermented KGB** *A. niger* and *A. oryzae* are generally considered safe nontoxic fungi and play important roles in producing various fermented foods.19) However, it was reported that certain strains of *A. niger* could produce ochratoxin and fumonisin while some strains of *A. oryzae* could produce aflatoxin and cyclopiazonic acid.20) In the present study, these mycotoxins were not detected in the fermented KGB (data not shown).

**Transformation of Ginsenosides by *A. niger* and *A. oryzae*** PPT type ginsenoside Re is the most plentiful ginsenoside in unfermented KGB. Ginsenoside Rb1, Rb2, and Rd are also contained as the main PPD type ginsenosides of unfermented KGB. After fermentation, the ginsenoside profile was analyzed by TLC (Fig. 1) and HPLC (Fig. 2). The percentage weight of various ginsenosides in the *n*-butanol extracts was determined by HPLC (Fig. 3).

In terms of TLC results (Fig. 1), bands of ginsenosides on the plate went up after fermentation. Furthermore, bands of cK and Rh1 markedly appeared on the top of columns of *niger* and *oryzae*-fermented KGB, respectively. These show that the major ginsenosides have been transformed to their less polar forms.

With respect to HPLC results (Fig. 3), after fermentation by *A. niger*, for the main PPT type ginsenosides, Re decreased from 17.8 to 0.2%, Rg2 increased from 1 to 6.5% and Rh1 increased from 0.1 to 3.3%. As for PPD type ginsenosides, Rbl, Rb2 and Rd decreased to 0% and cK increased from 0 to 10.3%. Thus it can be seen that *A. niger* mainly transformed PPT type ginsenoside Re to Rg2 and to a lesser extent, to Rh1. Most PPD type ginsenosides such as Rb1, Rb2, and Rd were transformed to cK in the end. In our previous research,15) ginsenoside Rbl was transformed to cK via Rd and F2 by crude enzyme extracted from various microorganisms, which is consistent with the present study. Ginsenoside Rg3 was also produced when KGB was sterilized by autoclave before fermentation and partly transformed to Rh2 by fermentation. These show that *A. niger* is more inclined to transform PPD type ginsenosides with a notably high cK yield while the PPT type ginsenoside transformation was stopped at ginsenoside Rg2, a less deglycosylated form.

After fermentation by *A. oryzae*, for the main PPT type ginsenosides, Re decreased from 17.8 to 0%, Rgl increased from 2.4 to 9.3% and Rh1 increased from 0.1 to 6.8%. As

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*Fig. 2. HPLC Chromatograms of Ginsenosides Detected from (A) Unfermented KGB, (B) *niger*-Fermented KGB, (C) *oryzae*-Fermented KGB*
for PPD type ginsenosides, Rb1 decreased from 1.2 to 0.5%, Rb2 decreased from 2.3 to 1.7%, and Rd decreased from 4.4 to 1.3%; F2 increased from 0 to 7.3%, and cK increased from 0 to 3.4% (Fig. 3). Thus it can be seen that A. oryzae transformed PPD type ginsenosides mainly to F2 and slightly to cK. Furthermore, ginsenoside Rg3 was also produced during sterilization. As for PPT type ginsenosides, Re was largely transformed to Rh1 via Rg1. Thus, it is apparent that A. oryzae may be more prone to transform PPT type ginsenosides with an efficient ginsenoside Rh1 production while the PPD type ginsenoside transformation was stopped at ginsenoside F2, a less deglycosylated form (Fig. 4).

Taken together, ginsenosides in ginseng berries were efficiently transformed into their non-polar forms. KGB fermented by A. niger showed high inhibitory effects on the HT-29 cell line while unfermented KGB showed no cytotoxicity at the tested concentration (Fig. 5A). The minimum effective concentration of niger-fermented KGB was less than 2.5 µg/mL while that of oryzae-fermented KGB ranged from 2.5 to 5 µg/mL. The IC50 of niger-fermented KGB was between 10 and 20 µg/mL while the oryzae-fermented KGB was between 5 and 10 µg/mL.

As for the HepG2 cell line, mold-fermented KGB also showed outstanding anti-proliferative activity while unfermented KGB only had a slight effect at concentrations larger than 20 µg/mL (Fig. 5B). The minimum effective concentration of niger-fermented KGB was less than 2.5 µg/mL while that of oryzae-fermented KGB ranged from 2.5 to 5 µg/mL. The IC50 value of niger-fermented KGB was about 10 µg/mL while that of oryzae-fermented KGB was almost 20 µg/mL.

To figure out which ginsenosides show cytotoxicity, PPD type ginsenoside Rb1, Rg3, cK, and PPT type ginsenosides Re and Rh1 were also tested by MTT assay (Fig. 5C). Rb1 showed no cytotoxicity at tested concentrations while its metabolized form, Rg3, exhibited the most efficient cytotoxicity. However, PPT type ginsenosides showed no cytotoxicity. As A. niger is more inclined to transform PPD type ginsenosides, niger-fermented KGB showed stronger anti-proliferative activity than oryzae-fermented KGB.

Additionally, according to the previous study,20) cytotoxic activity is inversely proportional to the numbers of sugars linked to the aglycone of ginsenosides. Steamed AGB was reported to exhibit enhanced cytotoxicity as a result of that its main PPD type ginsenosides were transformed to Rg3.6) How-

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**Fig. 3. Ginsenoside Contents in Butanol Extract of Unfermented and Fermented KGB Measured by HPLC**

Percentage concentration = (weight of ginsenoside/weight of sample) × 100%.

**Fig. 4. Proposed Main Transformation Pathways of Ginsenoside Re by Mycotoxin Non-producing A. niger and A. oryzae**
Cohort studies have reported that ingesting ginseng could diminish the risk of degenerative diseases including cancer.\(^{31-34}\) Case control and a clinical trial study indicated that Korean red ginseng powder might improve postoperative survival as well as restore immunity in patients with advanced gastric cancer.\(^{38}\)

The anti-cancer activity can be mainly ascribed to ginsenosides.\(^{34}\) Deglycosylated forms of PPD type ginsenosides such as cK have potent cytotoxicity and inhibit tumor cell invasion.\(^{39}\) Although PPT type ginsenosides have barely any cytotoxicity, they may also show curative effects against cancers. Protopanaxatriol was reported to inhibit the matrix metallopeptidase (MMP)-9 expression in the HT1080 human fibrosarcoma cell line and reduce the tumor cell invasion.\(^{40}\)

It has also been observed that the fatty acid conjugate form of panaxatriol activated natural killer (NK) cells so as to kill cancer cells in an indirect way.\(^{41}\) The PPT type ginsenosides have been documented to suppress glycoprotein dependent multidrug resistance and increase the sensitivity of cancer cells to anti-cancer drugs.\(^{42}\) In addition, PPT type ginsenoside metabolites have been shown to increase the effects of anti-cancer drug by inhibiting breast cancer resistance protein.\(^{43}\)

Fermentation by A. niger and A. oryzae dramatically increased cytotoxicity of KGB on the HT-29 and HepG2 cell lines, which in turn confirmed that ginsenosides in KGB were highly transformed. The minimum effective concentration was about 2 µg/mL, which can be easily reached in the gastrointestinal tract. Therefore, fermented KGB may show anti-proliferative effect on colon cancer cells, like HT-29 cells. However, according to the previous research, only nanogram levels of deglycosylated forms of ginsenosides were detected in the plasma after oral intake of ginseng products, indicating that it is hard to expect any cytotoxicity on cancer cells located beyond the gastrointestinal tract, like HepG2 cells.

In summary, our findings indicated that A. niger tended to transform PPD type ginsenosides and A. oryzae tended to transform PPT type ginsenosides. Contents of ginsenoside cK and Rh1 were increased by approximately 10 and 7% in A. niger and A. oryzae fermented KGB extracts, respectively. Moreover, after fermentation by these mycotoxin free molds, anti-proliferative activity of KGB was dramatically enhanced. As PPD type ginsenosides exhibit stronger cytotoxicity and A. niger are more prone to transform PPD type ginsenosides, A. niger fermented KGB showed more remarkable anti-proliferative effect. However, as the most ginsenosides in KGB are of PPT type, fermentation with A. oryzae may be more meaningful. Since berry and root have the different profiles of ginsenoside, they may exhibit distinct activities. It is reported that Rh1 and cK act as novel agonists of estrogen and glucocorticoid receptor, respectively.\(^{45,46}\) Dey et al.\(^{47}\) compared the anti-hyperglycemic effects between berry and root, and found that ginseng berry exhibited more potent anti-hyperglycemic activity, and only ginseng berry showed marked anti-obesity effects in ob/ob mice administrated intraperitoneally. More studies are needed to figure out the distinct activities between ginseng berry and root.

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**Conflict of Interest** Zhipeng Li, Hyung Jin Ahn, Nam Yeon Kim, and Yu Na Lee have no conflict of interest. Geun Eog Ji is a professor at Seoul National University, and also president of Bifido Co., Ltd.

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