Ginseng fermented by mycotoxin non-producing *Aspergillus niger*: ginsenoside analysis and anti-proliferative effects

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Abstract Korean ginseng was fermented using *Aspergillus niger* (A. niger) FMB S494 and mycotoxins such as ochratoxin and fumonisin were not detected in the fermented ginseng. Protopanaxadiol-type ginsenosides such as glycosylated forms of Rb1, Rb2, Rc, and Rd decreased to 0 while compound K (cK) increased from 0 to 9×10^4 ppm in the extract of fermented ginseng. Protopanaxatriol-type ginsenosides such as Re and Rg1 decreased from 7.1×10^4 to 3.0×10^4 ppm and 6.8×10^4 to 4.6×10^4 ppm, respectively. Rg2 and Rh1 increased from 0 to 1.9×10^4 ppm and 0 to 2.7×10^4 ppm, respectively. We can demonstrate that *A. niger* was more inclined to transform protopanaxadiol-type ginsenosides. Moreover, fermented ginseng extract showed a dramatically enhanced anti-proliferative effect on human HT-29 cell line with a minimum effective concentration of about 1 µg/mL, which might be attributed to the high degree of biotransformation of ginsenosides, especially the high output of ginsenoside cK.

Keywords Ginsenoside · *Aspergillus niger* · Biotransformation · Mycotoxin · Anti-proliferation

Introduction

Panax ginseng (C.A. Meyer, family Araliaceae) has been used as a kind of novel herb in East Asia for thousands of years. Ginsenoside, the active component of ginseng, can be divided into two groups: protopanaxadiol (PPD)-type and protopanaxatriol (PPT)-type. Structures of various PPD-type and PPT-type ginsenosides studied in this research are presented in Table 1. The biological activities of ginsenosides are based on the number and position of sugar moieties linked to the aglycone [1]. Actually, when ginseng was administered to human subjects, usually only the deglycosylated forms such as compound K (cK) and Rh1 were detected from the circulating system [2]. The deglycosylation degree of ginsenosides may be one of the reasons why commercial ginseng products vary in effects. After administrated orally, ginsenosides are transformed to their deglycosylated forms by intestinal flora. Different transformation ability of microflora may be one of the reasons why the effects of ginseng vary among individuals [3]. As a consequence, it may be necessary to transform ginsenosides before intake.

Various methods have been used to transform ginsenosides. Steaming [4], acid hydrolysis [5] and alkaline cleavage [6] are limited because they can hardly transform ginsenosides completely and they also cause inevitable side reactions such as epimerization, hydroxylation and hydration. Enzymatic conversion is also limited because it is complex and inefficient and can be hardly used in foods [7]. Therefore, microbial conversion, or fermentation, is in the limelight because it is handy and considered safe. However, of particular relevance is the fact that the microorganism to be used must be of a food-grade standard. Many researchers have reported during the past few decades that ginsenosides can be successfully transformed

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Table 1 Chemical structures of various ginsenosides

	R1	R2	R3
20(S)-Protopanaxadiol-type			
Rb1	O-Glc(2-1)Glc ^a	H	O-Glc(6-1)Glc
Rb2	O-Glc(2-1)Glc	H	O-Glc(6-1)Arap ^b
Rc	O-Glc(2-1)Glc	H	O-Glc(6-1)Arap
Rd	O-Glc(2-1)Glc	H	O-Glc
Rg3	O-Glc	H	OH
F2	O-Glc	H	O-Glc
cK	OH	H	O-Glc
Rh2	O-Glc	H	OH
20(S)-Protopanaxatriol-type			
Re	OH	O-Glc(2-1)Rha ^c	O-Glc
Rg1	OH	O-Glc	O-Glc
Rg2	OH	O-Glc(2-1)Rha	OH
Rh1	OH	OH	O-Glc

^a β-D-glucopyranosyl
^b α-L-arabinopyranosyl
^c α-L-rhamnopyranosyl

by human intestinal bacteria [8, 9]. However, these intestinal bacteria requires expensive medium and exhibits low yield and poor productivity [10]. There are also other researchers utilizing microorganisms isolated from soil-cultivated ginseng to transform ginsenosides [11, 12]. Nevertheless, soil microorganisms can be applied to foods only if they have been proved to be safe. Fungus is long used in traditional fermented foods because of its strong viability and saccharifying power. In this study, we used mycotoxin free *Aspergillus niger* to ferment Korean ginseng (KG) and observed the changes of the ginsenoside profile and the anti-proliferative activities.

Materials and methods

Microbial cultures and chemicals

4-year Korean ginseng (*Panax ginseng* C.A. Meyer, family Araliaceae) roots were purchased from Nokdu Market, Seoul, Korea, in March 2015. *Aspergillus niger* (FMB S494) was from Lab. of Food Microbiology, Seoul National University. Human colorectal adenocarcinoma HT-29 cell line was purchased from the Korean Cell Line

Bank. Standard ginsenosides such as Rb1, Rb2, Rd, and Rg1 were purchased from Biotech (Nanjing, China). Ginsenosides Re, Rg2, Rg3, F2, Rh1, Rh2, and cK were purchased from Cogon Biotech (Chengdu, China).

Fermentation of ginseng

Dried ginseng (50 g) was ground and extracted with 750 mL of distilled water at 80 °C with shaking. After filtration with 4 layers of gauze, the filtrate was used as culture broth. The *A. niger* was inoculated on PDA medium plate and cultured for 5 days at 30 °C under aerobic conditions. Then the spores were scraped from the plate and suspended in saline containing 0.005% Tween 80. *Aspergillus niger* was inoculated into the prepared broth at a density of 10⁶ spores/mL and cultured at 30 °C for 6 days under aerobic conditions with shaking.

Analysis methods

Fermented broth was tested if it contained mycotoxin ochratoxin A, fumonisin by the HPLC method according to the previous studies [13, 14].

After 6 days of fermentation, the culture broth was freeze-dried and the total dry mass was 14.1 g. After the dry matter was extracted with water-saturated n-butanol at 80 °C and filtrated with Whatman No. 41 filter paper (Kent, UK), the filtrate was mixed with distilled water and stewed overnight. The upper phase was evaporated with a speed vacuum concentrator and the left solid content was suspended in diethyl ether and maintained at 46 °C for 1 h so as to remove the fat-soluble impurities. After centrifuge, the supernatant was discarded and the remaining crude saponin was 1.4 g. Content of each ginsenoside was measured using HPLC according to a previous study [15].

Analysis of anti-proliferative effect

HT-29 cells were cultured in DMEM containing 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic antimycotic solution at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The anti-proliferative effects of crude saponin extracted from KG or FKG on HT-29 cell line were analyzed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After seeded in 96-well plates at a density of 2×10^4 cells per well and cultured for 24 h, cells were treated with various concentrations of crude saponin dissolved in DMSO for 48 h. Then, the supernatants were discarded and 50 mL MTT solution (1 mg/mL) was added to each well. After cells were cultured for another 2 h, the supernatant was again discarded and 200 μ L DMSO was added to each well and mixed well with shaking for 10 min. Absorbance at 490 nm was determined by a microplate reader (Philadelphia, PA).

Statistical analysis

Results were presented as mean $\pm$ SD. Differences were examined with analysis of variance followed by the least significance difference test with SPSS 21.0. The significance level was set at $p < 0.05$.

Results and discussion

Aspergillus niger is prevalently used in many fermented traditional foods and generally considered safe. However, a recent study suggests that certain strains of *A. niger* could produce ochratoxin and fumonisin [16]. In this study, these mycotoxins were not detected (data not shown).

According to the results of HPLC (Fig. 1), the main ginsenosides of unfermented KG are Rb1, Rb2, Rc, Rd, Re, Rf and Rg1, which is in line with the previous study [17]. After fermentation by *A. niger*, according to the quantitative analysis with HPLC (Fig. 2), PPD-type ginsenosides such as Rb1, Rb2, Rc and Rd decreased from 9.3×10^4 to 0 ppm, 3.7×10^4 to 0 ppm, 2.5×10^4 to 0 ppm and 5.0×10^4 to 0 ppm, respectively. Rg3, F2, Rh2 and cK increased from 0.9×10^4 to 4.9×10^4 ppm, 0 to 1.4×10^4 ppm, 0 to 2.9×10^4 ppm and 0 to 9.0×10^4 ppm, respectively. PPT-type ginsenosides such as Re, Rg1 and Rf decreased from 7.1×10^4 to 3.0×10^4 ppm, 6.8×10^4 to 4.6×10^4 ppm and 4.3×10^4 to 2.5×10^4 ppm, respectively. Rg2 and Rh1 increased from 0 to 1.9×10^4 ppm and 0 to 2.7×10^4 ppm, respectively.

Fig. 1 HPLC chromatograms of ginsenosides detected from crude saponin extracted from KG or FKG. (A) KG; (B) FKG

Fig. 2 Percentage concentration of ginsenosides determined by HPLC in crude saponin extracted from KG or FKG

In this work, we found a mold, *A. niger*, which can transform ginsenosides quite effectively. After fermentation, the contents of major ginsenosides such as Rb1, Rb2, Rc and Rd decreased to 0 and minor ginsenosides such as cK increased from 0 to 9.0×10^4 ppm in the extracts. Moreover, *A. niger* does not produce mycotoxins and can be considered safe. More importantly, during fermentation, substances such as carbon source and nitrogen source were not added to the culture broth. The whole fermentation process was simple to operate and the cost was low.

In summary, *A. niger* efficiently transformed PPD-type ginsenosides to cK and moderately transformed PPT-type ginsenosides to Rh1. Moreover, *A. niger* was more apt to transform PPD-type ginsenosides than the PPT-type with a dramatic increase of cK to 9.0×10^4 ppm, which was consistent with our previous study [15]. Ginsenoside cK was reported to have many biological and pharmaceutical effects such as anti-inflammation [18], anti-obesity [19], anti-diabetes [20] and anti-cancer [21].

Anti-proliferative effects of extracts of KG and fermented Korean ginseng (FKG) were analyzed by MTT assay. KG showed no anti-proliferative effect at concentrations up to 40 $\mu\text{g/mL}$, while FKG showed an outstanding effect even at an extremely low concentration of 1.25 $\mu\text{g/mL}$ (Fig. 3A). Fermentation dramatically increased the anti-proliferative effect of KG on HT-29 cells, which might be attributed to the change of ginsenoside profiles. According to a previous study [22], the cytotoxic effect of ginsenosides is inversely related to the sugar numbers and PPD type has higher activities than PPT type. As shown in Fig. 3, glycosylated forms of ginsenosides (i.e., Rb1) were transformed to their less glycosylated forms (i.e., cK). Anti-proliferative effects of Rb1 and cK were also tested and the results showed that Rb1 exhibited no anti-proliferative effect at the tested concentration while cK exhibited a significant effect at the concentration of 5 μM (3 $\mu\text{g/mL}$), which might provide insight into explaining why fermentation enhanced the effect of KG

Fig. 3 Anti-proliferative effects of KG and FKG (A), ginsenoside Rb1 and cK (B) on HT-29 cells. Proliferation of control group was set as 100%. Proliferation after treatment = Abs after treatment/Abs of control $\times 100\%$. * $p < 0.5$ versus control

(Fig. 3B). Nevertheless, of particular relevance is the fact that FKG extract even showed a stronger effect than ginsenoside cK, which demonstrates that there is probably a complicated synergistic effect among various ginsenosides that needs further research. As have been reported in previous studies [23, 24], PPT-type ginsenosides suppressed glycoprotein dependent multidrug resistance and increased the sensitivity of cancer cells to anticancer drugs.

In conclusion, *A. niger* FMB S494, a mycotoxin non-producing mold, efficiently transformed PPD-type ginsenosides of KG to their less glycosylated forms with a high yield of ginsenoside cK. Moreover, FKG showed a dramatically enhanced anti-proliferative effect on human HT-29 cells with a minimum effective concentration of 1.25 µg/mL, which might be attributed to the high yield of ginsenoside cK. Ginseng fermented by *A. niger* FMB S494 may be developed as a novel health food.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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