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Bioconversion of Ginsenosides in the American Ginseng (西洋參 Xī Yáng Shēn) Extraction Residue by Fermentation with Lingzhi (靈芝 Líng Zhī, *Ganoderma Lucidum*)

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ABSTRACT

Ginseng (人參 Rén Shēn) has been widely employed in functional foods and traditional medicines in many Asian countries. Owing to the high consumer demand of ginseng products, a large amount of ginseng residue is generated after extraction of ginseng. However, the ginseng residue still contains many bioactive compounds such as ginsenosides. The objective of this research was to convert ginsenosides in American ginseng (西洋參 Xī Yáng Shēn) extraction residue (AmR) by fermentation with lingzhi (靈芝 Líng Zhī, *Ganoderma lucidum*) and the fermentation products will be used for further hypoglycemic activity research. Thus, this study was primarily focused on the ginsenosides that have been reported to possess hypoglycemic activity. In this study, the changes in seven ginsenoside [Rg1, Re, Rb1, Rc, Rg3(S), compound K (CK), and Rh2(S)] in the products as affected by fermentation were investigated. Our results showed that the levels of ginsenosides, namely, Rg1, Rg3(S), and CK increased, while the other ginsenosides (Re, Rb1, and Rc) decreased during the fermentation process.

Key words: Bioconversion, *Ganoderma lucidum*, Ginseng, Ginsenoside, Lingzhi

INTRODUCTION

Ginseng (人參 Rén Shēn) possesses several beneficial effects in the prevention of diabetes, cancer, and cardiovascular disease,[1-3] and therefore, it is extensively used in different therapeutic and health-promoting preparations. These biological activities in ginseng may be attributed to the presence of bioactive compounds such as ginsenosides, polysaccharides, and flavonoids.[4] Among these bioactive compounds, ginsenosides have been extensively investigated as different ginsenosides possess varying pharmacological and biological activities. For instance, Rg1 and compound K (CK) were shown to stimulate glucose uptake in 3T3-L1 adipocytes,[5] with the former one increasing the performance of learning/memory in a mice model as well.[6] Furthermore, the composition of ginsenosides is the major indicator of ginseng quality and species type as the amount and type of ginsenosides in ginseng vary depending on the species, age, harvest season, growth condition, and processing methods.[4,7,8] The ratio of ginsenoside Rb1/Rg1 in American ginseng (西洋參 Xī Yáng Shēn) was reported to be higher than in Asian ginseng (東洋參 Dōng Yáng Shēn). Despite its potential health benefits, ginsenosides suffer from poor bioavailability. Previous studies have pointed out that the conversion of ginsenoside Rb1 into its deglycosylated metabolite CK could substantially increase both bioavailability and bioactivity,[9,10] with their bioavailability being 0.3%-1.2% and 35.0%, respectively.[11-14] Thus, it is important

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to convert the major ginsenoside Rb1 into its minor metabolites for improved bioactivity and bioavailability.

Lingzhi (靈芝 Líng Zhī, *Ganoderma lucidum*) is a popular traditional Chinese medicine (TCM) belonging to white rot basidiomycete. Generally, *G. lucidum* is investigated for its pharmacological activities including hyperglycemia, hypertension, immunomodulatory, liver protection, and anti-tumor effects.[15-18] In addition, lignin-modifying enzymes synthesized by *G. lucidum* could degrade lignins, cellulose, and hemi-cellulose.[19] In this study, *G. lucidum* was employed to degrade lignin and cellulose in the cell walls of American ginseng residue (AmR) for bioconversion of ginsenosides. Several bioconversion methods in ginsenosides include heating, acid or alkaline hydrolysis, enzymatic and microbial conversion. Specifically, chemical conversion methods are not environment friendly and may cause poor selectivity and low efficiency, eventually reducing the biological activity of ginsenosides,[20-22] whereas enzymatic conversion method is highly selective and environmentally compatible, especially under mild reaction conditions. However, the enzymes currently employed are not stable enough for their usage in industries.[22] On the other hand, microbial conversion method is more advantageous as it is ecofriendly, economically viable, and can be scaled up for good reproducibility. Consequently, microbial conversion is the most desirable bioconversion method for industrial application.[23]

In many Asian countries, abundant amounts of ginseng residue are produced as waste byproduct every year owing to its large application in manufacturing functional food products. Ultimately, the efficient utilization of ginseng extraction residue for potential application in various fields is a subject of significant interest. Moreover, *G. lucidum* is also a valuable TCM and has been rarely investigated for bioconversion of ginsenosides. In this study, we intend to study the bioconversion of ginsenosides in AmR by *G. lucidum* and determine the changes in ginsenoside composition as affected by fermentation conditions of *G. lucidum* grown on AmR. This study can not only develop a valuable functional food ingredient by combining the benefits of both ginseng and lingzhi, but can also resolve the problem of ginseng waste management.

MATERIALS AND METHODS

Materials

American ginseng extraction residue was supplied by a local food company, while AmR fermentation products were prepared by Dr. Ting-Jang Lu of our institute using *G. lucidum* (BCRC37066) from Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu City, Taiwan). Ginsenoside standards including Re, Rg1, Rb1, Rc, Rg3(S), and Rh2(S) were purchased from Advantage Chemical Co. Ltd. (Taichung, Taiwan). Ginsenoside standard CK was obtained from Tauto Biotech Ltd. (Shanghai, China), digoxin from Sigma (St. Louis, MO, USA) and organic solvents acetonitrile, ethyl alcohol, and methyl alcohol from Mallinckrodt Baker (NJ, USA).

Instrumentation

Shaking water bath (model 905) was from Hotech (Taipei, Taiwan), rotary evaporator (RE111) from Buchi (Flawil, Switzerland), centrifugal vacuum concentrator (SCV100H) from Savant (Farmingdale, NY, USA), ultrasonic processor (S4000) from Misonix (Farmingdale, NY, USA), and centrifuge (model 2100) from KUBOTA (Tokyo, Japan).

Fermentation method

The fermentation method included two steps. The first step was inoculum preparation. *G. lucidum* was cultivated in malt extract agar with 2% ginseng residue at 25°C for 7 days. Afterward, *G. lucidum* was harvested and then seeded in AmR at 1% w/w (low inoculation), 5% w/w (medium inoculation), and 10% (w/w) (high inoculation) of inoculum quantity for subsequent incubation at 25°C for 4, 8, or 13 days.

Sample preparation

A method based on Wang, *et al*.,[24] was modified for extraction of ginsenosides from unfermented AmR and *G. lucidum* fermented AmR (FAmR). One gram sample was mixed with 20 mL of 80% methanol–water solution at a sample to solvent ratio of 1:20 and shaken for 1 h at 50°C, followed by filtering through a Whatman No. 1 filter paper, concentrating under vacuum at 40°C, and freeze-drying. Next, 50 mg of the extract was dissolved in 1 mL of deionized water and purified by using Sep-Pak C18 Cartridge (Phenomenex, Inc., Torrance, CA, USA) by activating sequentially with 5 mL each of methanol and deionized water. The extract solution (1 mL) was then passed through the C18 Cartridge, washed with 5 mL of deionized water, and finally eluted with 5 mL of methanol. The eluted methanol extract was evaporated to dryness under vacuum, the residue redissolved in 1 mL of methanol, filtered through a 0.2 μm membrane filter (Chrom Tech, Inc., Minnesota, USA), and 10 μL injected into high-performance liquid chromatography (HPLC) for analysis.

HPLC analysis of ginsenosides

For the separation of various ginsenosides in unfermented AmR and FAmR, a method based on Kim *et al*.,[25] was modified and used. A Hitachi HPLC system (Toyko, Japan) consisted of a chromatographic pump (L-7100), autosampler (L-7200), and UV-VIS detector (L-7420). A C18 Atlantis column (4.6 mm ID × 150 mm, 3 μm particle size) from Waters and a binary solvent system of deionized water (A) and acetonitrile (B) was used for separation with the following gradient conditions: 79% A and 21% B initially, increased to 22% B at 6 min, 23% B at 7 min, 24% B at 25 min, 30% B at 30 min, 32% B at 40 min, 50% B at 45 min, 65% B at 60 min, 100% B at 61 min, and maintained until 71 min. The flow rate was maintained at 0.8 mL/min, column temperature at 30°C, and detection wavelength at 203 nm.

Quantification of ginsenosides in ginseng residue and its fermentation products

The quantification of ginsenosides in unfermented AmR and FAmR samples was conducted by incorporating an internal standard into the ginsenoside standard solution and developing standard curves based on the peak area ratio versus the concen-

tration ratio. Digoxin was selected as the internal standard and a concentration of 100 μM was prepared in methanol. The concentration range of 0.25-400 μM for each ginsenoside standard Rg1, Re, Rb1, Rc, Rg3(S), CK, and Rh2(S) was prepared separately and mixed with digoxin for a final concentration of 60 μM. The calibration curves were plotted by standard peak area (As)/internal standard peak area (Ai) versus standard concentration (Cs)/internal standard concentration (Ci) in μM and then submitted to linear regression analysis in Microsoft Excel software to obtain regression equation for each ginsenoside. The quantity of each ginsenoside was calculated by the regression equation.

Determination of recovery

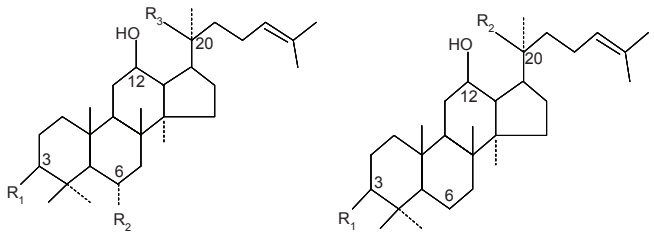
Different amounts of each ginsenoside standard were added separately to ginseng waste powder and then subjected to extraction and HPLC analysis. The recovery of each ginsenoside standard was calculated based on the ratio of the amount initially added to that determined after HPLC analysis. The recovery of each ginsenoside was 96.5 ± 0.71% for Rg1, 89.5 ± 3.54% for Re, 84.5 ± 2.12% for Rb1, 92.5 ± 10.61% for Rc, 87.5 ± 6.36% for Rg3, 93 ± 1.41% for CK, and 81 ± 2.38% for Rh2.

Statistical analysis

Each measurement was conducted in triplicate and the results were evaluated by analysis of variance (ANOVA) and Duncan's multiple range test for significance in mean comparison ($P = 0.05$) by using the SAS software system.

RESULTS AND DISCUSSION

The structure of ginsenosides is shown in Figure 1. In this study, we primarily focused on the ginsenosides that have been reported to possess hypoglycemic activity and the changes in these ginsenosides during fermentation. Seven ginsenosides, namely, Rg1, Re, Rb1, Rc, Rg3(S), CK, and Rh2(S), were analyzed in ginsenoside standards and fermentation samples by using HPLC. Figures 2 and 3 depict the HPLC chromatogram for separation of ginsenosides in ginsenoside standards mixture and unfermented AmR as well as FAmR samples on different days of fermentation, respectively. Our results showed that the content of ginsenosides Rb1, Re, and Rc in AmR was higher than the other ginsenosides [Table 1 and Figure 4], with their levels amounting to 49.66, 23.61, and 15.05 μmol/g, respectively. However, Ligor *et al*.,[26] have reported the amount of Rb1 to be significantly higher than the other ginsenosides in American ginseng, and this contrasting result may be due to variation in the sample variety. In the absence of any fermentation, the total content of ginsenosides Rb1, Re, and Rc in unfermented AmR was 90.83 μmol/g, and after fermentation with *G. lucidum* for 13 days, the amount diminished to a same level of 11.06 μmol/g for both 1% low and 5% medium inoculations and to 10.84 μmol/g for 10% high inoculation. However, compared to without fermentation (1.72 μmol/g), the total amount of other three ginsenosides Rg1, Rg3(S), and CK soared, attaining a level of 5.33, 6.07, and 5.06 μmol/g for 1%, 5%, and 10% inoculations, respectively. The results showed that the total amount of seven ginsenosides was dramatically decreased during fermentation, which might be attributed to the reason that some major transformed ginsenosides in the fermentation products were not analyzed.



Protopanaxadiol Protopanaxatriol

Compound	R1	R2	R3
Protopanaxadiol			
Rb1	-O-Glc^2-Glc	-O-Glc^6-Glc	-H
Rc	-O-Glc^2-Glc	-O-Glc^6-Araf	-H
Rg3	-O-Glc^2-Glc	-O-H	-H
Rh2	-O-Glc	-O-H	-H
Compound K	-O-H	-O-Glc	-H
Protopanaxatriol			
Re	-O-H	-O-Glc^2-Rha	-O-Glc
Rg1	-O-H	-O-Glc	-O-Glc

Araf: α-L-arabinose (furanose); Glc: β-D-glucose; Rha: α-L-rhamnose

Figure 1. The chemical structures of ginsenosides

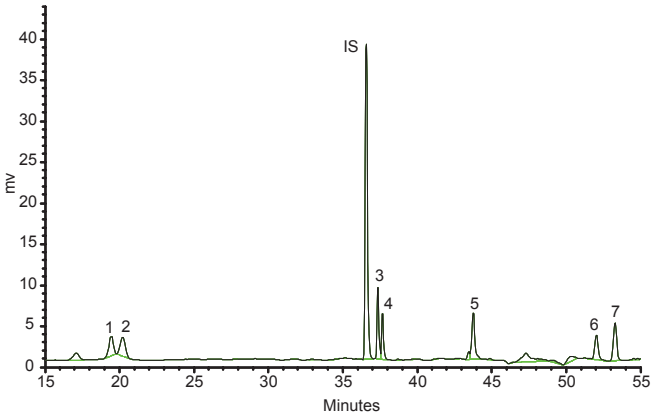


Figure 2. HPLC chromatogram for separation of seven ginsenoside standards mixed with an internal standard digoxin (IS): 1, Rg1; 2, Re; 3, Rb1; 4, Rc; 5, Rg3(s); 6, CK; 7, Rh2(s). The concentration of each ginsenoside standard was 50 μM, while that of IS digoxin was 60 μM. HPLC conditions are described in the section Materials and Methods

For protopanaxadiol type ginsenosides, the ginsenoside Rb1 levels in unfermented AmR, FAmR at 1% inoculation, FAmR at 5% inoculation, and FAmR at 10% inoculation were 49.66, 8.99, 10.08, and 8.40 μmol/g, respectively, while much lower values of 15.05, 1.42, 0.86, and 1.70 μmol/g were obtained for ginsenoside Rc, indicating a decreasing trend in the content of Rb1 and Rc during fermentation. On the contrary, both Rg3(S) and CK increased upon fermentation with their levels equaling 1.57 and 0.15 μmol/g for unfermented AmR, 4.00 and 0.05 μmol/g for FAmR at 1% inoculation, 3.21 and 0.36 μmol/g for FAmR at 5% inoculation, and 2.76 and 0.37 μmol/g for FAmR at 10% inoculation, respectively. This observed trend in ginsenoside levels during fermentation

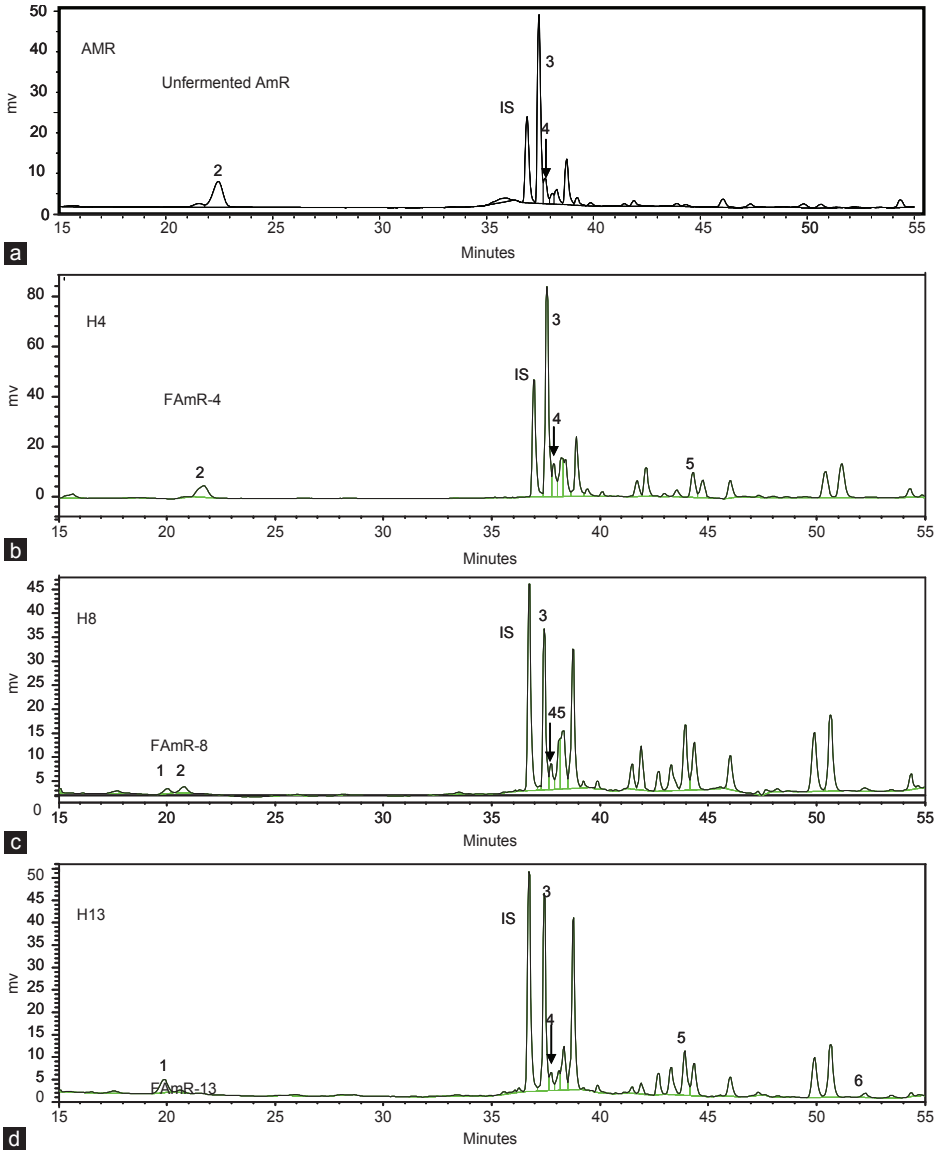


Figure 3. HPLC chromatogram for separation of ginsenosides in unfermented American ginseng residue (AmR) and *G. lucidum* fermented AmR (FAmR). (a) AmR without fermentation; (b) FAmR-4, AmR fermented with *G. lucidum* at 10% inoculation for 4 days; (c) FAmR-8, AmR fermented with *G. lucidum* at 10% inoculation for 8 days; (d) FAmR-13, AmR fermented with *G. lucidum* at 10% inoculation for 13 days. 1, Rg1; 2, Re; 3, Rb1; 4, Rc; 5, Rg3(s); 6, CK. Sample concentration, 2000 μg/mL; IS concentration, 60 μM

may be caused by the conversion of ginsenoside Rb1 into Rg3 by removal of two glucose moieties from position C-20 and Rb1 into CK by elimination of two glucose units from C-3 followed by one glucose from C-20 position. Such conversions were reported by Takino[27] as well as Qian and Cai,[28] with the former demonstrating the transformation of Rb1 into its deglycosylated product Rg3 after incubation with 0.1 M HCl at 37°C, while the same conversion was shown in gastrointestinal tract by the latter after oral administration of Rb1 to rats. Additionally, deglycosylation and oxygenation were identified as the two major metabolic pathways responsible for conversion of Rb1 into its metabolites. A bioconversion of Rb1 into CK by a β-glucosidase active bacterium *Leuconostoc citreum* LH1 was also reported by Quan, *et al*.[29] Likewise, conversion of Rc into Rg3 and CK can also occur by removal of one glucose and one arabinose from C-20 position for the former and elimination of two glucose units from C-3 followed by one arabinose from C-20 for the latter. A fungus-mediated bioconversion of Rc into CK by *Fusarium sacchari* was reported by Han, *et al*.[30] Thus, the increase in ginsenoside Rg3 and CK levels may be caused by bioconversion of Rb1 and Rc during fermentation of AmR with *G. lucidum*.

Upon fermentation for 13 days, the level of protopanaxatriol type ginsenoside Re significantly dropped, with its content in unfermented AmR, FAmR at 1% inoculation, FAmR at 5% inoculation, and FAmR at 10% inoculation amounting to 23.61, 0.40, 0.00, and 0.20 μmol/g, respectively. The ginsenoside Rg1 was undetected in unfermented AmR; however, its amount rose to 1.27, 2.49, and 1.93 μmol/g during fermentation with 1%, 5%, and 10% inoculations, respectively. Theoretically, ginsenoside Re can be converted into Rg1 by removal of one rhamnose from C-6

Table 1. Changes in ginsenoside content during the fermentation of American ginseng residues (AmR) with *G. lucidum*

	Bioconverted ginsenosides (μmol/g of sample)								
	AmR[b]	FAmR at 1% low inoculation[c]			FAmR at 5% medium inoculation[c]			FAmR at 10% high inoculation[c]		
		D4[d]	D8[d]	D13[d]	D4[d]	D8[d]	D13[d]	D4[d]	D8[d]	D13[d]
Rg1	0.00±0.00	0.00±0.00	1.47±2.08	1.27±1.71	0.00±0.00	0.03±0.05	2.49±0.86*	0.39±0.671	0.39±0.17	1.93±0.44**
Re	23.61±0.50	2.42±0.60**	0.80±1.12**	0.40±0.56***	2.13±0.67***	1.16±0.62***	0.00±0.01***	3.10±0.45***	0.67±0.23***	0.20±0.17***
Rb1	49.66±0.01	12.49±0.49**	11.55±0.17***	8.99±3.77**	11.68±2.98***	11.85±3.98***	10.08±0.70***	17.62±2.76***	8.70±0.60**	8.40±3.43***
Rc	15.05±4.05	2.87±0.49	2.38±1.55	1.42±1.21*	2.90±0.93*	3.02±0.78*	0.86±0.33**	5.10±0.99*	3.78±2.49	1.70±1.64*
Rg3 (S)	1.57±0.30	2.29±0.11	2.91±0.42	4.00±0.55*	2.53±0.80	4.49±0.94*	3.21±0.32*	3.19±0.07**	4.73±0.15**	2.76±1.03
CK	0.15±0.03	0.00±0.00*	0.01±0.02*	0.05±0.07	0.00±0.00**	0.11±0.10	0.36±0.16	0.00±0.00**	0.09±0.11	0.37±0.26
Rh2 (S)	2.51±0.17	0.00±0.00**	0.38±0.54*	0.24±0.34*	0.00±0.00***	0.20±0.34**	0.11±0.20**	0.00±0.00***	0.04±0.06**	0.54±0.93
Totala	92.55±4.06	20.07±1.69**	19.50±0.67**	16.39±3.97**	19.24±5.31***	20.85±5.92***	17.12±1.24***	29.40±4.07***	18.42±3.29**	15.90±6.13**
Rg1+Rg3+CK (Percentage to total ginsenosides amount)	1.72 (1.86)	2.29 (11.41)	4.39 (22.51)	5.33 (32.52)	2.53 (13.15)	4.62 (22.16)	6.07 (35.46)	3.58 (12.18)	5.22 (28.34)	5.06 (31.82)
Re+Rb1+Rc+Rh2 (Percentage to total ginsenosides amount)	90.83 (98.14)	17.79 (88.59)	15.11 (77.49)	11.06 (67.48)	16.71 (86.85)	16.23 (77.84)	11.06 (64.54)	25.82 (87.82)	13.20 (71.66)	10.84 (68.18)

Data are shown as mean±SD (n=3). *indicated significant difference (P<0.05) from AmR by Student's t test. **indicated significant difference (P<0.01) from AmR by Student's t test. ***indicated significant difference (P<0.001) from AmR by Student's t test. [a]Total represents the sum of seven ginsenosides, [b]AmR represents unfermented American ginseng residues, [c]Fermented American ginseng residues at different percentage inoculations (1, 5, and 10%) and percentage inoculation represents the quantity of *G. lucidum* inoculated into the American ginseng residue, [d]D4-D13 represent the fermentation time in days.

Figure 4. Changes in ginsenoside content during fermentation of American ginseng residues with *G. lucidum* at (a) 1% low inoculation, (b) 5% medium inoculation, and (c) 10% high inoculation

position. Therefore, we suggest that the increase in Rg1 level in FAmR samples may be caused by conversion of Re during fermentation of AmR. Besides ginsenosides, the lingzhi triterpenes, including ganoderic acid A, B, C2, D, E, F, G, and F, were also analyzed. However, all of these ganoderic acids were not detected in the fermentation samples. This might be attributed to the insufficient fermentation time for ganoderic acid production (data not shown). Additionally, the lingzhi bioactive polysaccharide maker (1,3)-β-d-glucan, which is a large molecule, also increased during fermentation (unpublished data). Thus, the fermentation products combined the benefits of both ginseng and *G. lucidum*.

The major ginsenosides Rg1 and Rg3(S) found in FAmR samples had been shown to possess several biological activities. In several studies, Rg1 has been demonstrated to possess hypoglycemic activity and increase the performance of learning/memory as well in mice model.[5,6,31] Nevertheless, vasodilating, hepatoprotective, and neuroprotective effects were reported for ginsenoside

Rg3.[32-34] Interestingly, a higher pharmacological activity was shown by minor ginsenosides like Rg3 and CK when compared to the major ones (Rb1, Rc, and Re).[20,35] However, the amount of Rg3 is extremely low in normal ginseng, and hence, it is important to develop methods to convert major ginsenosides like Rb1 into minor Rg3 for good biological activity.[36] Among several conversion methods, enzymatic and microbial conversions are often preferred for production of minor ginsenosides. Using an enzymatic conversion method, Quan, *et al*.,[37] converted the ginsenoside Rb1 into Rg3 by employing a recombinant β-glucosidase (Bgp1) from *Microbacterium esteraromaticum*. In two different studies involving microbial conversion method, *Microbacterium* sp. (GS514 strain) and *Acremonium strictum* were used for bioconversion of Rg3.[36,38] Likewise, Chi and Ji[39] have utilized the fungus *Aspergillus niger* for microbial bioconversion of Re into Rg1. Actually, in microbial conversion methods, microorganisms are cultivated in liquid medium by reacting directly with the substrate (ginsenoside Rb1). However, the ginsenoside substrate is expensive and these microbial methods require further purification of ginsenoside products. Thus, microbial conversion methods may not be economically viable and practically feasible especially for application in industries. In this study, AmR was used as the fermentation medium for *G. lucidum* which degrades the cell wall of AmR first and, thus, may not react directly with the substrate (ginsenoside Rb1). Despite the long time duration required by this method, the fermentation products obtained could be utilized directly without further purification, thereby making our bioconversion method both economically and practically feasible for developing valuable new functional food ingredient by combining the benefits of both ginseng and *G. lucidum*. It is worth pointing out that *G. lucidum* is rarely used for the bioconversion of ginsenosides and utilization of ginseng residue for functional food development would be a "wealth from waste," besides solving the problem of waste disposal. This is only a preliminary study to investigate the bioconversion of ginsenosides in the AmR by lingzhi (*G. lucidum*) and further research is necessary to elucidate the detailed bioconversion procedure.

CONCLUSION

An eco-friendly and economically viable microbial fermentation method was developed for bioconversion of ginsenosides in American ginseng extraction residue into more bioactive fermentation products. Upon fermentation with *G. lucidum*, the amount of ginsenosides Re, Rb1, and Rc declined, while Rg1 and Rg3(S) increased. Thus, this study could not only develop a valuable new functional food ingredient by combining the benefit of both ginseng and lingzhi, but also resolved the problem of ginseng waste management.

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