



Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: <http://www.ginsengres.org>

Research article

Biotransformation of *Panax ginseng* extract by rat intestinal microflora: identification and quantification of metabolites using liquid chromatography-tandem mass spectrometry



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ARTICLE INFO

Article history:

Received 18 March 2016

Received in Revised form

6 October 2016

Accepted 21 November 2016

Available online 5 January 2017

Keywords:

ginsenoside

LC–MS/MS

metabolites

Panax ginseng

rat intestinal microflora

ABSTRACT

Background: In general, after *Panax ginseng* is administered orally, intestinal microbes play a crucial role in its degradation and metabolization process. Studies on the metabolism of *P. ginseng* by microflora are important for obtaining a better understanding of their biological effects.

Methods: *In vitro* biotransformation of *P. ginseng* extract by rat intestinal microflora was investigated at 37°C for 24 h, and the simultaneous determination of the metabolites and metabolic profile of *P. ginseng* saponins by rat intestinal microflora was achieved using LC–MS/MS.

Results: A total of seven ginsenosides were detected in the *P. ginseng* extract, including ginsenosides Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd. In the transformed *P. ginseng* samples, considerable amounts of deglycosylated metabolite compound K and Rh1 were detected. In addition, minimal amounts of deglycosylated metabolites (ginsenosides Rg2, F1, F2, Rg3, and protopanaxatriol-type ginsenosides) and untransformed ginsenosides Re, Rg1, and Rd were detected at 24 h. The results indicated that the primary metabolites are compound K and Rh1, and the protopanaxadiol-type ginsenosides were more easily metabolized than protopanaxatriol-type ginsenosides.

Conclusion: This is the first report of the identification and quantification of the metabolism and metabolic profile of *P. ginseng* extract in rat intestinal microflora using LC–MS/MS. The current study provided new insights for studying the metabolism and active metabolites of *P. ginseng*.

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1. Introduction

Panax ginseng Meyer, which is a well-known plant medicine with diverse pharmacological effects, has been used as a functional food or dietary supplement in China, Korea, and Japan. As a Traditional Chinese medicine, ginseng is widely used as a single herb or in herbal mixtures to promote health and treat diseases [1]. The main active components of ginseng are ginsenosides.

The structures of the ginsenoside monomers of more than 50 species have been isolated and identified. Ginsenosides are connected by an aglycone and a sugar and belong to the triterpenoid saponin group. Based on the structure of the aglycone, the

ginsenosides can be divided into three categories as follows: oleanolic acid-type ginsenosides, protopanaxadiol (PPD)-type ginsenosides, and protopanaxatriol (PPT)-type ginsenosides. The PPD-type or PPT-type ginsenosides have different sugar moieties at positions C-3/C-6 and C-20 in the aglycon PPD or PPT (Fig. 1). The ginsenosides exhibit various biological activities including anti-inflammatory activity, anticancer activity, reduce blood pressure and cardiovascular system, immunomodulatory effects, and antioxidant activities [2–6]. Intestinal microflora is an important “microflora organ,” and in both human and animals, approximately 99% of the intestinal bacteria are anaerobic bacteria. In humans, the intestinal microbiota consists of more than 10¹⁴ bacteria and

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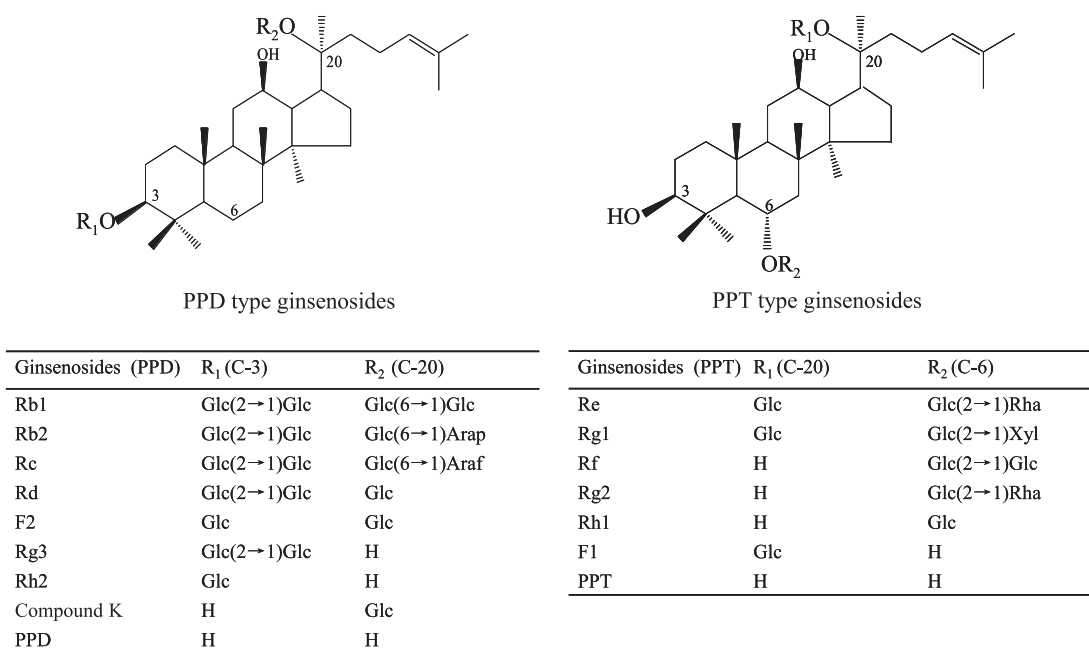


Fig. 1. Chemical structures of protopanaxadiol (PPD)-type ginsenosides and protopanaxatriol (PPT)-type ginsenosides *Glc* β -D-glucopyranosyl, *Arap* α -L-arabinopyranosyl, *Araf* α -L-arabinofuranosyl, *Xyl* β -D-xylopyranosyl and *Rha* α -L-rhamnopyranosyl.

archaea, which include approximately 1,100 prevalent species [7–9]. It plays an important role in the metabolism of compounds that are administered orally [10].

In addition to its medicinal usage, *P. ginseng* is also widely consumed as a food in the form of tea, powder, drinks, liquid

extracts, candy, and capsules. Therefore, the components of orally administered *P. ginseng* products inevitably come into contact with intestinal microbiota in the digestive tract [11,12]. During this process, the major ginsenosides of orally administered *P. ginseng* are biotransformed to minor ginsenosides followed by absorption.

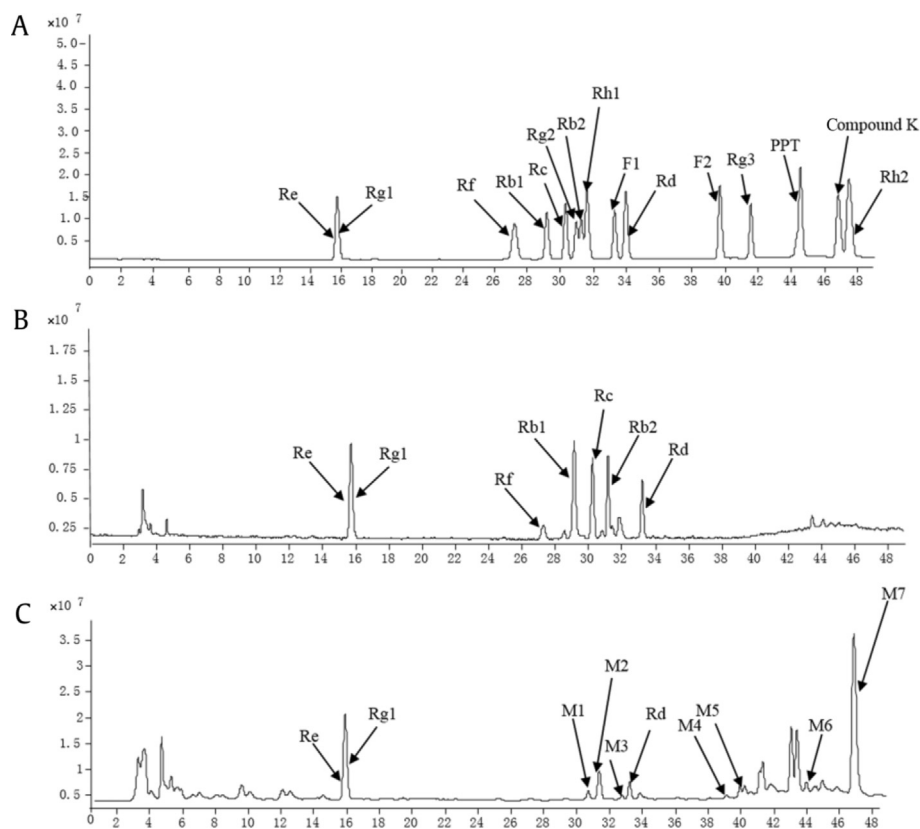


Fig. 2. The typical total ion chromatogram of ginsenosides in the positive-ion mode by LC–MS/MS. (A) mixed standards; (B) *Panax ginseng* extract; (C) transformed *P. ginseng* extract by rat intestinal microflora within 24 h.

Table 1
LC–MS/MS data in the positive-ion mode of standard ginsenosides.

Ginsenoside	Rt (min)	Formula	Formula (mass)	(m/z)		Product ions (m/z)	Collision energy
				[M+Na] ⁺	[M-2H ₂ O+H] ⁺		
Rg1	15.8	C ₄₂ H ₇₂ O ₁₄	800.0	823.4	—	643.6 [M-glc-H ₂ O+Na] ⁺ 203.0 [glc+H ₂ O+Na] ⁺	40
Re	15.8	C ₄₈ H ₈₂ O ₁₈	946.0	969.5	—	789.2 [M-glc-H ₂ O+Na] ⁺ 203.0 [glc+H ₂ O+Na] ⁺	50
Rf	27.0	C ₄₂ H ₇₂ O ₁₄	800.0	823.3	—	481.0 [M-(glc+glc)-H ₂ O+Na] ⁺ 365.0 [(glc+glc)+H ₂ O+Na] ⁺	60
Rb1	29.0	C ₅₄ H ₉₂ O ₂₃	1108.0	1131.8	—	789.8 [M-(glc+glc)-H ₂ O+Na] ⁺ 365.3 [(glc+glc)+H ₂ O+Na] ⁺	60
Rc	30.2	C ₅₃ H ₉₀ O ₂₂	1078.0	1101.7	—	790.7 [M-(glc+ara)-H ₂ O+Na] ⁺ 335.2 [(glc+ara)+H ₂ O+Na] ⁺	60
Rb2	31.2	C ₅₃ H ₉₀ O ₂₂	1078.0	1101.5	—	789.5 [M-(glc+ara)-H ₂ O+Na] ⁺ 335.2 [(glc+ara)+H ₂ O+Na] ⁺	60
Rd	33.2	C ₄₈ H ₈₂ O ₁₈	946.0	969.5	—	789.5 [M-glc-H ₂ O+Na] ⁺ 365.3 [(glc+glc)+H ₂ O+Na] ⁺ 203.0 [glc+H ₂ O+Na] ⁺	50
Rg2	30.8	C ₄₂ H ₇₂ O ₁₃	784.0	807.5	—	481.0 [M-(rha+glc)-H ₂ O+Na] ⁺ 349.2 [(rha+glc)+H ₂ O+Na] ⁺	56
Rh1	31.4	C ₃₆ H ₆₂ O ₉	638.0	—	603.0	423.3 [M-glc-H ₂ O+H] ⁺	10
F1	33.9	C ₃₆ H ₅₈ O ₉	638.0	661.4	—	481.2 [M-glc-H ₂ O+Na] ⁺ 202.9 [glc+H ₂ O+Na] ⁺	34
F2	39.8	C ₄₂ H ₇₂ O ₁₃	784.0	807.3	—	627.2 [M-glc-H ₂ O+Na] ⁺ 203.0 [glc+H ₂ O+Na] ⁺	40
Rg3	41.6	C ₄₂ H ₇₂ O ₁₃	784.0	807.5	—	465.0 [M-(glc+glc)-H ₂ O+Na] ⁺ 365.0 [(glc+glc)+H ₂ O+Na] ⁺	67
PPT	44.5	C ₃₀ H ₅₂ O ₄	476.0	—	441.0	423.3 [M-3H ₂ O+H] ⁺	10
Compound K	46.9	C ₃₆ H ₆₂ O ₈	622.0	645.6	—	465.2 [M-glc-H ₂ O+Na] ⁺ 203.0 [glc+H ₂ O+Na] ⁺	30
Rh2	47.5	C ₃₆ H ₆₂ O ₈	622.0	—	587.0	407 [M-glc-H ₂ O+H] ⁺	10

Therefore, the characterization of the metabolism of *P. ginseng* saponins by intestinal microflora is important for elucidating the pharmacological actions of *P. ginseng* [13–15]. Many attempts have employed modern approaches for studying these important ginsenosides including the metabolism of some ginsenosides (Rb1, Rb2, Rc, Rd, Ra1, and Ra2) bioactivated by intestinal bacteria [16–21]. However, most of these studies only investigated a single ginsenoside or one intestinal bacteria strain but the metabolism of *P. ginseng* extract by synergetic intestinal microflora have been ignored. Due to competitive effects and exposure to compounds with different concentrations, the administration of a single ginsenoside from a *P. ginseng* extract may alter the metabolic profile [1].

Studies on the metabolism of *P. ginseng* by microflora are important for obtaining a better understanding of their

biological effects. In this study, an *in vitro* biotransformation study of *P. ginseng* extract with rat intestinal microflora was performed, and LC-MS/MS was used to identify and quantify the metabolites of *P. ginseng* saponins. It investigated the comprehensive metabolites and metabolic profile of *P. ginseng* extract.

2. Materials and methods

2.1. Chemicals and materials

The standard ginsenosides, Rg1, Re, Rf, Rb1, Rc, Rb2, Rd, Rg2, Rh1, F1, F2, Rg3, PPT, compound K, and Rh2 were purchased from the National Institute for the Control of Pharmaceutical and

Table 2
Validation of the method for the LC–MS/MS determination of ginsenosides. LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation

Ginsenosides	Calibration curve equation	Correlation coefficient	Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Recovery % (RSD%)		
						100 ng/mL	1,000 ng/mL	2,000 ng/mL
Re	y = 10.691x + 2519.7	R ² = 0.9973	50–5,000	7.51	24.99	104.4 (8.8)	94.8 (6.6)	116.5 (7.4)
Rg1	y = 13.074x + 7896.7	R ² = 0.9930	50–5,000	4.52	15.06	104.2 (11.1)	116.8 (10.1)	104.2 (8.9)
Rf	y = 9.7808x + 4535.2	R ² = 0.9940	50–5,000	4.23	14.09	98.9 (8.9)	98.2 (4.8)	109.2 (9.4)
Rb1	y = 4.7875x + 360.18	R ² = 0.9964	50–5,000	12.01	40.03	97.6 (10.0)	101.9 (0.6)	106.2 (2.6)
Rc	y = 1.9243x + 520.16	R ² = 0.9951	50–5,000	15.05	50.17	95.2 (7.3)	95.1 (9.1)	100.4 (4.5)
Rb2	y = 1.6348x + 545.69	R ² = 0.9955	50–5,000	12.04	40.12	90.4 (5.0)	80.1 (8.8)	109.1 (8.7)
Rd	y = 10.886x + 1745.9	R ² = 0.9917	50–5,000	2.27	7.56	104.9 (6.9)	101.7 (4.1)	95.5 (3.9)
Rg2	y = 0.5688x + 389.94	R ² = 0.9902	50–5,000	20.05	66.83	115.8 (9.6)	94.5 (10.7)	111.7 (7.7)
Rh1	y = 2.6693x + 170.07	R ² = 0.9987	50–5,000	3.33	11.11	123.7 (9.5)	107.7 (8.1)	107.2 (2.2)
F1	y = 4.189 x + 1495.3	R ² = 0.9905	50–5,000	9.38	31.25	96.1 (8.1)	100.5 (0.4)	95.9 (4.9)
F2	y = 11.384x + 4093.2	R ² = 0.9910	50–5,000	2.64	8.79	104.9 (6.5)	109.5 (11.5)	107.2 (2.3)
Rg3	y = 11.374x + 3971.8	R ² = 0.9916	50–5,000	13.63	45.43	112.0 (9.2)	99.2 (10.1)	105.2 (7.6)
PPT	y = 5.7564x + 1116.6	R ² = 0.9971	50–5,000	7.23	24.09	109.3 (4.8)	86.5 (7.6)	102.9 (0.9)
Compound K	y = 6.5315x + 1551.7	R ² = 0.9953	50–5,000	5.35	17.83	78.5 (8.2)	101.4 (9.6)	82.5 (8.8)
Rh2	y = 5.5618x + 1891.3	R ² = 0.9978	50–5,000	4.68	13.59	82.0 (9.2)	99.5 (8.7)	113.7 (9.1)

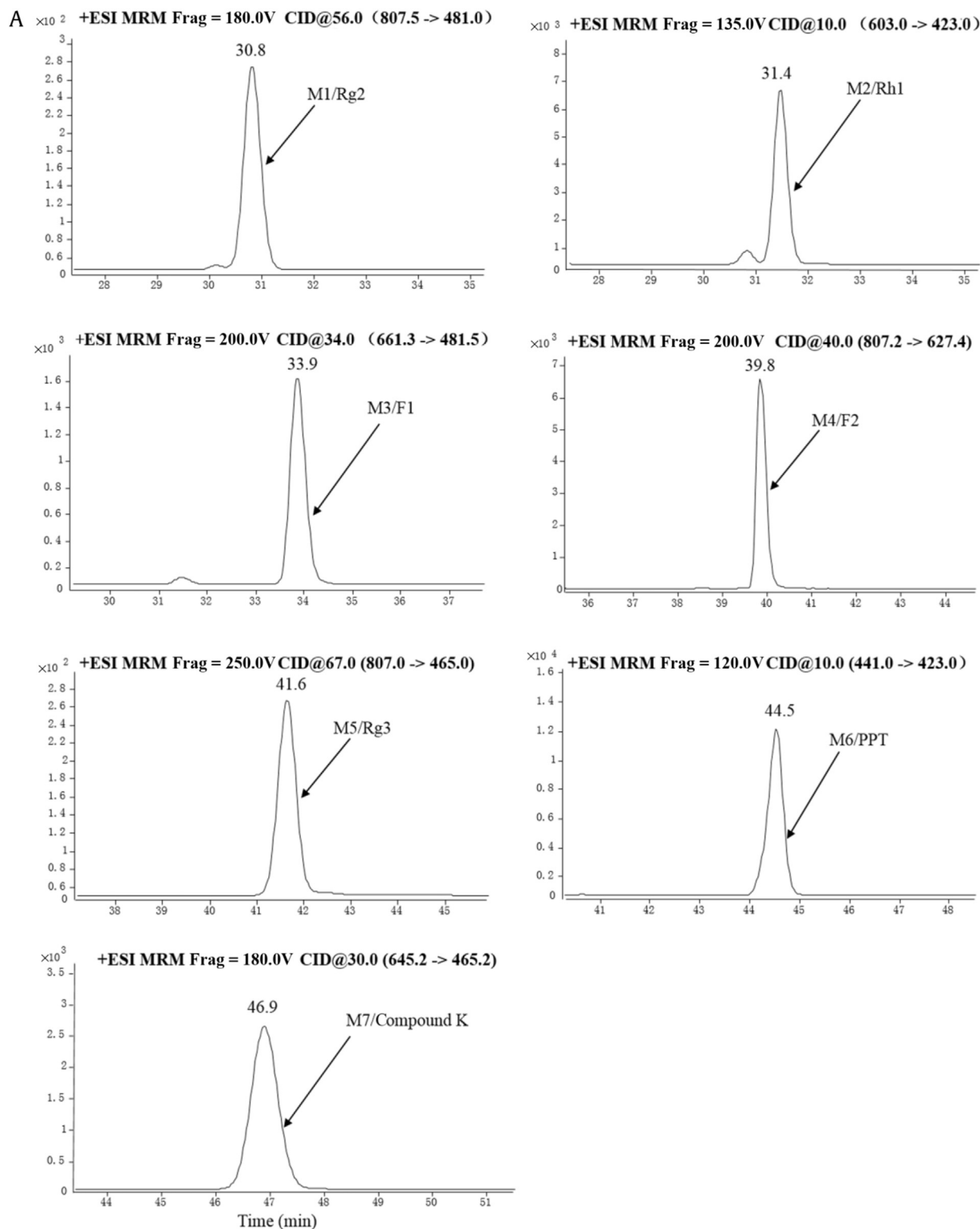


Fig. 3A. The representative multiple reaction monitoring (MRM) chromatograms of metabolites in the transformed (A) *Panax ginseng* sample and the (B) MS/MS fragmentation spectrum of the deglycosylated metabolites using electrospray ionization–MS/MS (ESI–MS/MS).

Biological Products (Beijing, China). General anaerobic medium broth was purchased from Changchun Sai wei-si Biological Technology Co., Ltd. (Changchun, China). HPLC grade methanol (MeOH) and acetonitrile were purchased from J.T. Baker (Center Valley, PA, USA).

2.2. *P. ginseng* extract preparation

P. ginseng roots (4 yr old) were obtained from JiAn, Jilin, China. The *P. ginseng* samples were pulverized into powder. One-hundred grams of the powder was extracted in an ultrasonic bath with 1 L of

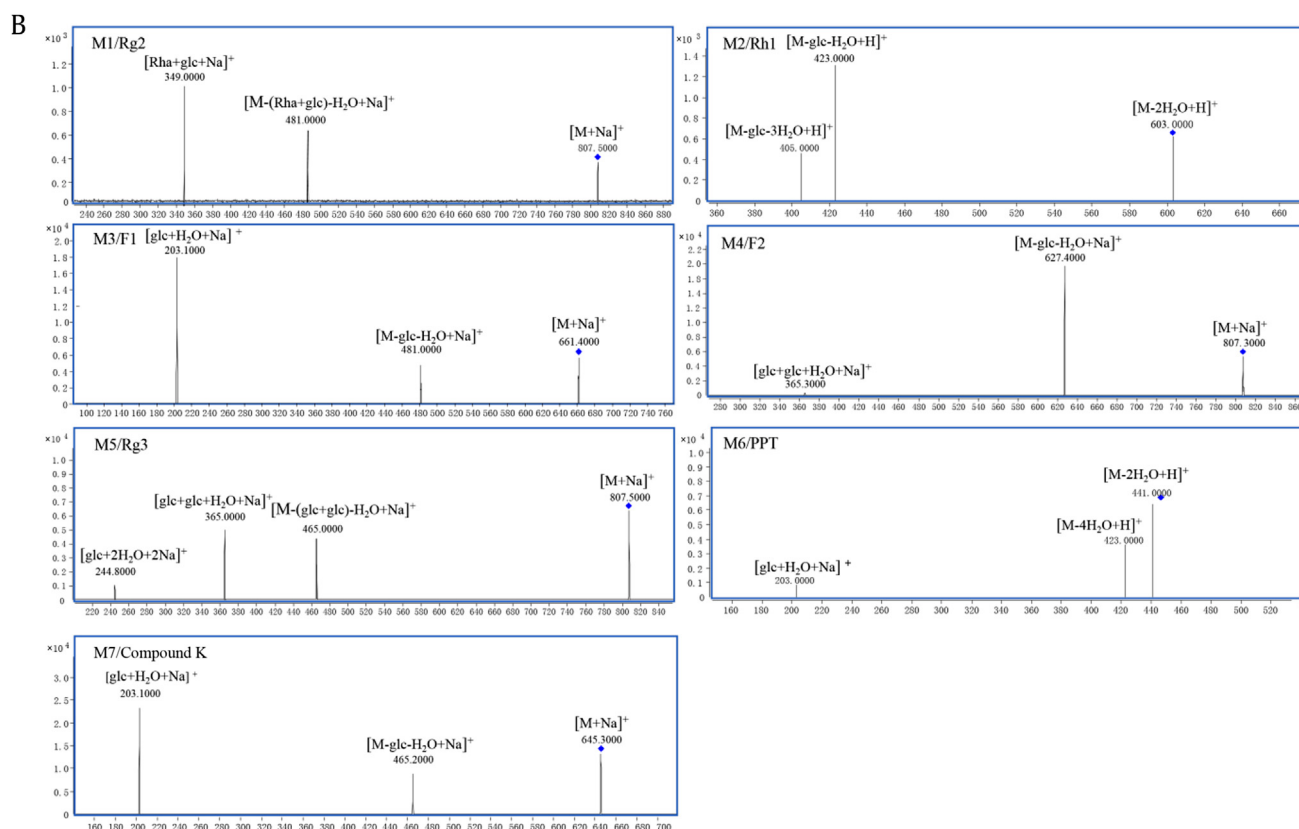


Fig. 3B. (continued)

80% ethanol at room temperature for 1 h. The operation was repeated twice, and the combined extracts were evaporated under vacuum and lyophilized.

2.3. Rat intestinal microflora sample preparation

Adult male Sprague-Dawley rats (Changchun) weighing 220–240 g were used in this study ($n = 5$). All animals were acclimated for more than 1 wk under a 12 h light and 12 h dark cycle at a room temperature of $23 \pm 1^\circ\text{C}$. Animal welfare and experimental procedures were conducted in strict accordance with the guidelines for the Yanbian University Animal Care Committee.

The rat colonic contents samples (1 g) were suspended in 9 mL of cold physiological saline. The fecal suspension was centrifuged at 500g for 5 min, and then, the supernatant was centrifuged at 10,000g for 20 min. The resulting precipitates were suspended in 15 mL of general anaerobic medium broth and anaerobically (85% N₂, 10% H₂, 5% CO₂) incubated at 37°C for 48 h.

2.4. Biotransformation of *P. ginseng* extract by rat intestinal microflora

In a previous study of metabolism of ginsenosides by intestinal microflora *in vivo* and *in vitro*, major ginsenosides have been reported to be mostly transformed into deglycosylated ginsenosides in 24 h [1,22,23]. In this study, 1 mL of the intestinal microbiota was added to 4 mg of the *P. ginseng* extract and anaerobically incubated at 37°C for 24 h. The reaction mixtures were extracted three times with four volumes of water-saturated *n*-BuOH. The *n*-BuOH layers were combined and dried by a speed vacuum followed by dilution to the desired volume with methanol. Each sample was filtered through a 0.2- μm filter prior to LC–MS/MS analysis.

2.5. LC–MS/MS analysis of ginsenosides

Chromatographic analysis was performed using an Agilent 1260 series LC system (Agilent Technologies, Palo Alto in California, US) equipped with a binary pump, an online degasser, an auto plate sampler, and a thermostatically-controlled column compartment. The system was operated using Mass Hunter Acquisition Software, Version B.07.00 (Agilent Technologies).

Sample separation was achieved on an Agilent Poroshell ZORBAX SB-C18 (Agilent Technologies) column (4.6 mm \times 150 mm, 5 μm) with a constant flow rate of 0.5 mL/min at 25°C. The mobile phase was consisted of water (A) and acetonitrile (B), and the following gradient elution was employed: 23–30% (B) for 0–15 min, 30–44% (B) for 15–34 min, 44–68% (B) for 34–46 min, 68–85% (B) for 46–61 min, 85–80% (B) for 61–66 min, 80–40% (B) for 66–71 min, and 40–20% (B) for 71–73 min. The injected sample volume was 2 μL .

Positive electrospray ionization–MS–MS was employed to analyze the metabolites of ginsenosides. All of the mass spectrometric experiments were performed on a triple-quadrupole tandem mass spectrometer (Agilent Technologies). The capillary voltage was set to 4,000 V for positive mode, and the gas flowed at a rate of 3 L/min with a gas temperature of 300°C. Full-scan mass spectra in a mass range of m/z 400–1,500 were acquired. The masses of the positive ion fragments [M+Na]⁺ or [M-2H₂O+H]⁺ of the potential metabolites were examined to determine the ginsenoside metabolites.

2.6. Validation of quantitative ability

The quantitative analysis consisted of an external calibration method. A stock solution containing 15 reference compounds were

prepared and diluted to appropriate concentrations for construction of calibration curves. The calibration curves were generated by plotting the peak area versus the concentrations of each analytes. The limits of detection that produced a signal-to-noise ratio of 3 and quantification under the present chromatographic conditions produced a signal-to-noise ratio ≥ 10 . Each standard was prepared in triplicate.

The recovery of this method was achieved using the standard addition method. Three different concentration levels (100 ng/mL, 1,000 ng/mL, 2,000 ng/mL) of the references standards were added into the sample in triplicate. The average recoveries were determined by the following formula: Recovery (%) = (observed amount – original amount)/spiked amount $\times 100\%$, with relative standard deviation (%) = (standard deviation/mean) $\times 100\%$.

3. Results and discussion

3.1. Characterization of standard ginsenosides

We analyzed 15 reference ginsenoside standards using LC-MS/MS. Fig. 2A shows the typical total ion chromatogram (TIC) of the

extract in positive ion mode. Table 1 shows the mass spectrometric conditions and fragmentation behaviors with data in the MS/MS stage of ginsenosides and its metabolites.

3.2. Method validation of LC-MS/MS

The method exhibited a good linear response over concentration range using linear regression analysis. Linear calibration curves with correlation coefficients greater than 0.99 were obtained for all analytes in the concentration ranges 50–5,000 ng/mL. The results proved that this technique is capable of generating good reproducibility (data shown in Table 2). The recoveries of the investigated targets ranged from 78.5% to 123.7% and the relative standard deviation of most compounds was less than 15%.

3.3. Characterization of ginsenosides in the *P. ginseng* extract

We analyzed the *P. ginseng* root (4 yr old) sample by LC-MS/MS. The TIC of the *P. ginseng* extract in positive ion mode is shown in Fig. 2B. The seven major ginsenosides were analyzed. By comparing

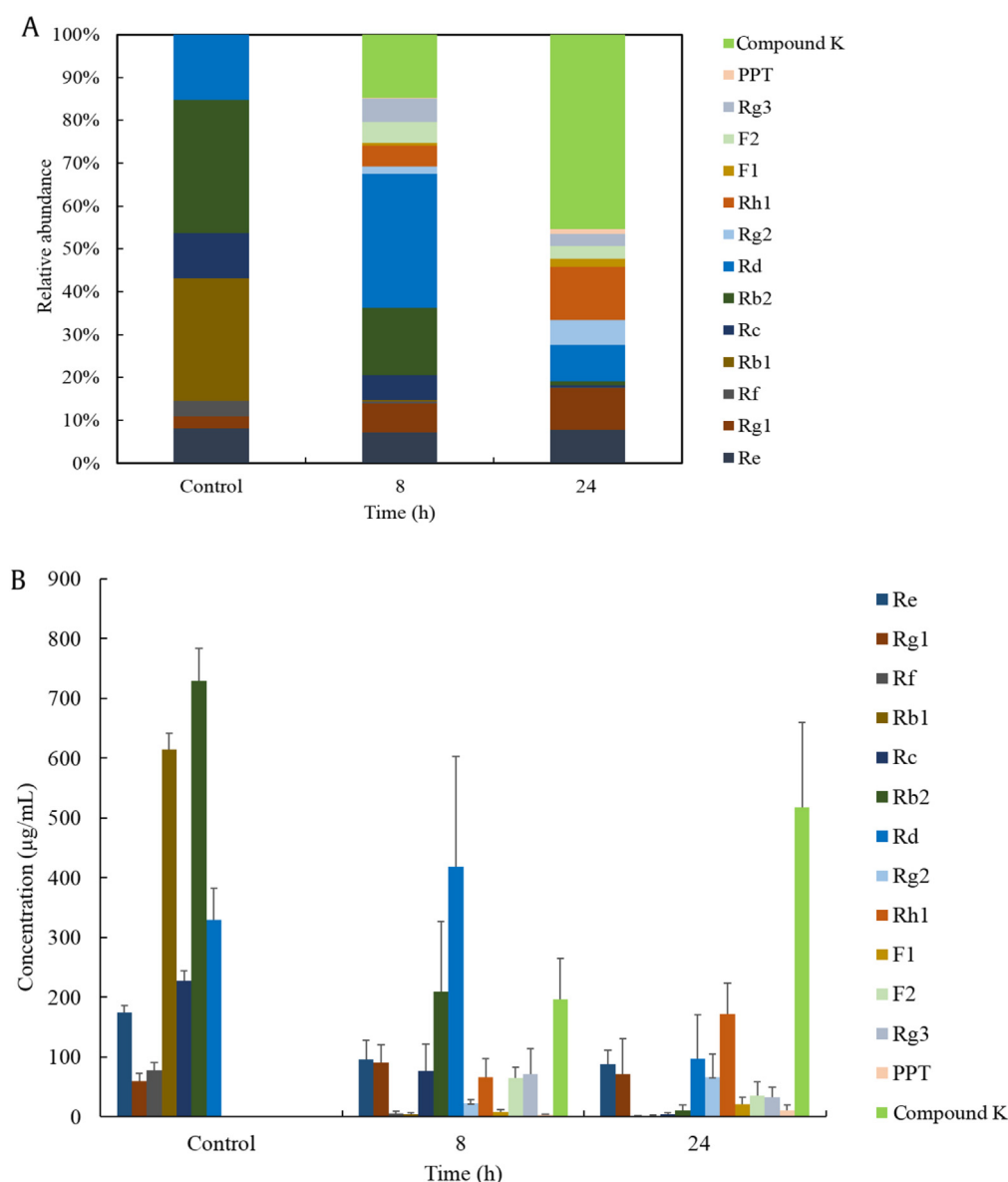


Fig. 4. The (A) relative abundance and (B) concentration-time profiles of ginsenosides in the transformed *Panax ginseng* extract by rat intestinal microflora.

the retention times, accurate masses, and characteristic MS/MS fragment ions of *P. ginseng*, the ginsenosides in the extract were identified. The seven major ginsenosides were determined to be ginsenosides Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd.

3.4. Identification and quantification of metabolites of *P. ginseng*

To understand the role of intestinal microflora in the metabolism of *P. ginseng* in the intestinal tract, rat intestinal microflora were employed to study the *in vitro* biotransformation of the *P. ginseng* extract. Fig. 2C shows the TIC of the transformed *P. ginseng* sample. A total of seven metabolites were identified. The

representative multiple reaction monitoring chromatograms for the metabolites are shown in Fig. 3A, and the MS/MS fragmentation spectrum of the deglycosylated metabolites using electrospray ionization–MS/MS (Fig. 3B): Metabolites M1 (30.8 min) at m/z 807.5 → 481.0, M2 (31.4 min) at m/z 603.0 → 423.0, M3 (33.9 min) at m/z 661.3 → 481.5, M4 (39.8 min) at m/z 807.2 → 627.4, M5 (41.6 min) at m/z 807.0 → 465.0, M6 (44.5 min) at m/z 441.0 → 423.0 and M7 (46.9 min) at m/z 645.2 → 465.2 were detected in the transformed *P. ginseng* sample. From comparison of the retention times and molecular mass to those of standard compounds, metabolites M1, M2, M3, M4, M5, M6, and M7 were identified as ginsenosides Rg2, Rh1, F1, F2, Rg3, PPT, and compound K,

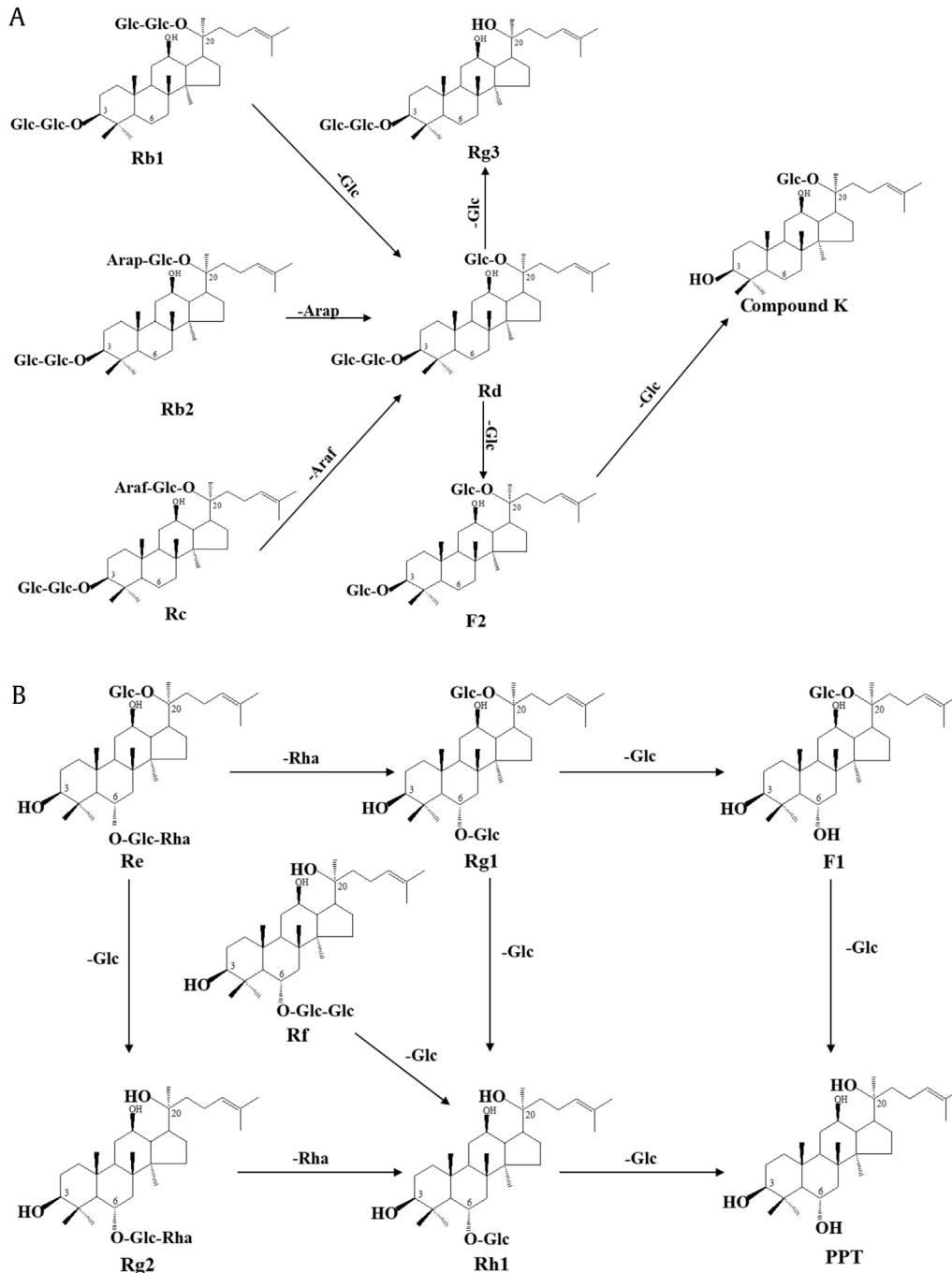


Fig. 5. Proposed deglycosylated metabolic pathway of *Panax ginseng* saponins by rat intestinal microflora. (A) protopanaxadiol (PPD)-type ginsenosides metabolized by rat intestinal microflora; (B) protopanaxatriol (PPT)-type ginsenosides metabolized by rat intestinal microflora.

respectively. Besides the metabolites determined, several peaks were also detected in the TIC. The work will be done in the future in our laboratory.

The biotransformation of *P. ginseng* extract by rat intestinal microflora was confirmed quantitatively by multiple reaction monitoring analysis. As shown in Fig. 4, considerable amounts of deglycosylated metabolite compound K and Rh1 were detected. In addition, minimal amounts of deglycosylated metabolites, such as ginsenosides Rg2, F1, F2, Rg3, and PPT as well as untransformed ginsenosides Re, Rg1, and Rd were detected in the transformed *P. ginseng* samples after 24 h. The results indicated that the primary metabolites are compound K and Rh1, and the PPD-type ginsenosides were more easily metabolized than PPT-type ginsenosides.

3.5. Metabolic pathways of *P. ginseng* saponins

The metabolic pathways of *P. ginseng* saponins include deglycosylation reactions by intestinal microflora through the selective elimination of sugar moieties. According to the metabolism of ginsenosides detected in this study and previous study of metabolic pathways consulted [24,25], we speculated the pathway of ginsenosides shown in Fig. 5. The metabolic pathways of PPD-type ginsenosides shown in Fig. 5A, illustrated the main metabolic pathways involve the selective elimination of the C-20 and C-3 outer sugar moieties to produce ginsenoside F2 from ginsenosides Rb1, Rb2, Rc and Rd, and then, C-3 sugar moieties to produce compound K. The metabolic pathways of the PPT-type ginsenosides via intestinal microflora are proposed in Fig. 5B. The metabolic pathway involves the elimination of the C-20 and C-6 sugar moieties to produce Rh1 or PPT.

4. Conclusion

We studied the *in-vitro* biotransformation of *P. ginseng* extract by rat intestinal microflora. A total of seven ginsenosides were detected in the *P. ginseng* extract including PPT-type ginsenosides Rg1, Re, and Rf, and PPD-type ginsenosides Rb1, Rc, Rb2, and Rd. In the transformed *P. ginseng* samples, considerable amounts of deglycosylated metabolite compound K and Rh1 were detected. In addition, minimal amounts of deglycosylated metabolite including ginsenosides Rg2, F1, F2, Rg3, and PPT as well as untransformed ginsenosides Re, Rg1, and Rd were detected after 24 h. The results indicated that the primary metabolites are compound K and Rh1, and the PPD-type ginsenosides were easily metabolized. This is the first report of the identification and quantification of the metabolism of *P. ginseng* extract by rat intestinal microflora. The current study provided new insights for studying the metabolism and active metabolites of *P. ginseng*.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

This study was supported by a China Postdoctoral Science Foundation funded project 287 (Number 2015M571376) and a grant from the National Natural Science Foundation of China (Number 81660643; 81603365).

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