Ginsenosides from Heat Processed Ginseng

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A new dammarane-glycoside, ginsenoside Rz_1 (1), was isolated from heat-treated *Panax ginseng* C. A. MEYER (Araliaceae) with ginsenosides Rk_1 and Rg_5. The structure of 1 was established to be (*Z*)-12β-hydroxy-dammara-20(22),24-dien-3β-yl *O*-β-D-glucopyranosyl-(1→2)-β-D-glucospyranoside by spectroscopic means. HPLC analyses revealed that the ginsenosides Rz_1, Rk_1, and Rg_5 were present in the ratios of 1 : 2 : 6, respectively.

Key words *Panax ginseng*; heat treatment; ginsenoside Rz_1; HPLC

The root of *Panax ginseng* C. A. MEYER (Araliaceae), commonly known as Korean ginseng, has several physiologic effects, *e.g.*, it has a tranquillizing action on the central nervous system, elevates blood pressure, and protects against physical and chemical stress.[1)] Extensive studies on this plant, including phytochemical and pharmacological approaches, have shown that its main bioactive principles are dammarane-type saponins.[2)] Furthermore, the saponins of *P. ginseng* have been shown to be panaxadiol and panaxatriol derivatives, and of the panaxadiol saponins present in heat-treated *P. ginseng* (red ginseng),[3)] ginsenoside Rg_3 is produced by the selective attack on the C-20 glycosidic bond during the steaming process.[4)] In particular, recent studies have showed that ginsenoside Rg_3 is converted to Rg_5 and Rk_1 by heat-treatment, and that these two saponins represent positional isomers of the double bond at C-20(21) or C-20(22),[4,5)] which is produced by E_1 elimination at C-20 of Rg_3 (Fig. 1).[6,7)]

The separation of the ginsenoside Rg_3 dehydrates, ginsenosides Rk_1 (**2**) and Rg_5 (**3**) has been achieved using conventional methods, such as column chromatography.[8,9)] However, these reported methods are inappropriate for determining the natures of the ginsenoside Rg_5 isomers at C-20(22) (**1**). Here, we report on the isolation and identification of compound **1**, and on the relative proportions of **1**, **2**, and **3** in manufactured red ginseng by HPLC/UV.

Results and Discussion

Repeat column chromatography of the butanol-soluble fraction of the MeOH extract of red ginseng roots using reversed-phase silica gel columns led to the isolation of three compounds (**1**—**3**). Two of these compounds were identified as ginsenosides Rk_1 (**2**) and Rg_5 (**3**), which had been previously isolated from red ginseng, based on their spectral and physical properties.[5)]

Compound **1** (ginsenoside Rz_1) was isolated as a white powder (MeOH), mp 188—191 °C, with negative optical rotation ($[\alpha]_D$ −10.5°). Electrospray ionization mass spectrometry (ESI-MS) spectrum exhibited a quasi-molecular ion peak at *m*/*z* 767 $[M+H]^+$, which corresponded to the dehydrated structure of ginsenoside Rg_3, such as ginsenosides Rk_1 (**2**) and Rg_5 (**3**). The molecular formula of **1** was deduced as $C_{42}H_{70}O_{12}$ on the basic of the peak at *m*/*z* 767.4908 $[M+H]^+$ from HR-ESI-MS. Characteristic ^1H- and ^{13}C-NMR signals

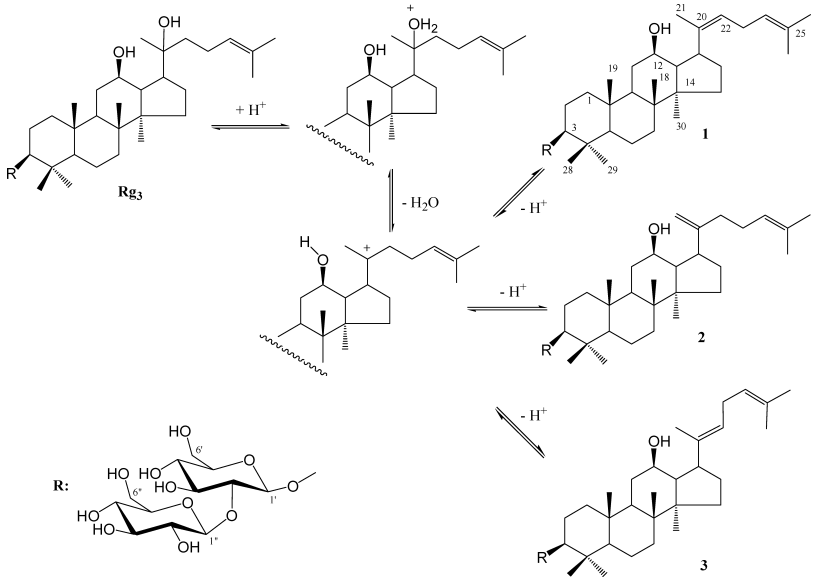

Fig. 1. Ginsenoside Rz_1 (**1**) and Other Two Conversion Products (**2**, **3**) of Ginsenoside Rg_3

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(Table 1) for methyl groups (δ_H 1.05×2, 1.95, 1.69, 1.67, 1.33, 1.14, 0.85) and (δ_C 16.3, 17.5, 33.1, 26.2, 18.2, 28.6, 17.1, 16.9), and olefinic peaks [δ_H 5.29 (1H, t, J=7.2 Hz), 5.31 (1H, t, J=9.0 Hz)] and (δ_C 140.3, 131.3, 125.0, 124.5), revealed **1** to be a dammarane derivative (Table 1). Two sugar units were easily discerned from its NMR spectra based on the low field signals of the anomeric protons and carbons [δ_H 4.96 (1H, d, J=7.2 Hz), 5.41 (1H, d, J=7.2 Hz)] and (δ_C 105.6, 106.6); other ^{13}C signals (δ_C 84.0, 78.8, 78.6, 78.4, 77.7, 72.1, 63.3, 63.1) revealed that **1** contained a sophorosyl [β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl] moiety. All ^{1}H-NMR data were based on C–H correlations of ^{1}H-detected multiple quantum coherence (HMQC). The four proton peaks at δ_H 1.48 (H-11a), 2.03 (H-11b), 1.70 (H-15a), and 1.08 (H-15b) correlated with δ_C 33.2 (C-11, 15). These NMR data were very similar to that of **3**. However, the obvious difference from **3** concerned the methyl carbon (C-21) signal at δ_C 33.1 in **1**. The ^{13}C chemical shift value of C-21 is *ca.* 13 ppm due to the presence of an *E*-type double-bond, as is present in the ginsenosides Rg_5, Rh_3, and Rh_4,[5] whereas in the case of a *Z*-type double-bond, this is expected at *ca.* 20—30 ppm.[10] The HMBC spectrum of **1** showed a long-range correlation between H-17 (δ_H 2.10), H-21 (δ_H 1.95), and H-22 (δ_H 5.29) and C-22 (δ_C 124.5) indicate for the 20(22)-ene group. In the nuclear Overhauser effect spectroscopy, a strong NOE correlation between H_3-21 and H-22 indicated a *Z*-orientation of C-20(22). Thus, the structure of compound **1** was elucidated to be (*Z*)-12β-hydroxydammara-20(22),24-dien-3β-yl *O*-β-D-glucopyranosyl-(1→2)-β-D-glucospyranoside and it was named ginsenoside Rz_1.

Table 1. NMR Data for Ginsenoside Rz_1 (**1**) in Pyridine-d_5

Carbon	δ_C [a]	δ_H [b]	Carbon	δ_C [a]	δ_H [b]
1	39.7 (CH_2)	1.53, 0.79	22	124.5 (CH)	5.29, t (7.2)
2	27.2 (CH_2)	1.84, 2.22	23	27.6 (CH_2)	3.00
3	89.4 (CH)	3.31, dd (7.2, 4.2)	24	125.0 (CH)	5.31, t (9.0)
4	40.2 (C)		25	131.3 (C)	
5	56.8 (CH)	0.72, d (11.4)	26	26.2 (CH_3)	1.69, s
6	18.9 (CH_2)	1.38, 1.52	27	18.2 (CH_3)	1.67, s
7	35.8 (CH_2)	1.25, 1.51	28	28.6 (CH_3)	1.33, s
8	40.7 (C)		29	17.1 (CH_3)	1.14, s
9	51.3 (CH)	1.45	30	16.9 (CH_3)	0.85, s
10	37.5 (C)		1′	105.6 (CH)	4.96, d (7.2)
11	33.2 (CH_2)	1.1—2.0	2′	84.0 (CH)	4.25, t (8.4)
12	73.0 (CH)	3.93	3′	78.8 (CH)	4.40
13	50.1 (CH)	3.62	4′	72.1 (CH)	4.38
14	51.7 (C)		5′	78.6 (CH)	4.28, t (7.2)
15	33.2 (CH_2)	1.1—2.0	6′	63.1 (CH_2)	4.51, br s
16	28.9 (CH_2)	1.70, 2.04	1″	106.6 (CH)	5.41, d (7.2)
17	51.0 (CH)	2.10, t (10.8)	2″	77.7 (CH)	4.17, t (8.4)
18	16.3 (CH_3)	1.05	3″	78.8 (CH)	3.96
19	17.5 (CH_3)	1.05	4″	72.1 (CH)	4.18, t (10.8)
20	140.4 (C)		5″	78.4 (CH)	4.33
21	33.1 (CH_3)	1.95	6″	63.3 (CH_2)	4.58, d (10.8)

a) Recorded at 75 MHz. *b*) Recorded at 600 MHz.

To select an optimal condition for the analysis of three structural isomers (ginsenosides Rz_1, Rk_1 and Rg_5) in red ginseng extracts, several HPLC runs at various levels of acetonitrile in water were performed. However, all conditions examined (column, solvent system, temperature, and flow-rate) failed to separate ginsenosides Rz_1 and Rk_1 (Fig. 2A). We then examined a double column extended. Attempts with a two extended C-18 column (Supelco C-18, 2.0×100 mm, 5 μm) using acetonitrile : water (45 : 55, 1.2 ml/min) as eluant successfully separated compounds **1**—**3** (Fig. 2B). In Fig. 2, chromatogram A shows a typical result obtained using standard one-column method for determining ginsenoside levels in red ginseng extract and chromatogram B shows that the double column extended separated the three isomers and neighboring components in red ginseng extract. The linear regression equations of the isomers were y=9992962x+108772 (**1**, r^2=0.9999), y=9893271x+100634 (**2**, r^2=0.9998) and y=9912736x+12926 (**3**, r^2=0.9999), respectively, where y=peak area and x=concentration (mg/ml). The detection limits were 0.11 μg/ml (**1**), 0.10 μg/ml (**2**) and 0.10 μg/ml (**3**) at a signal to nose ratio of **3**. The levels of compounds **1**—**3** in manufactured red ginseng extract ranged from 0.02 to 0.2% (mean 0.102%), 0.04 to 0.26% (mean

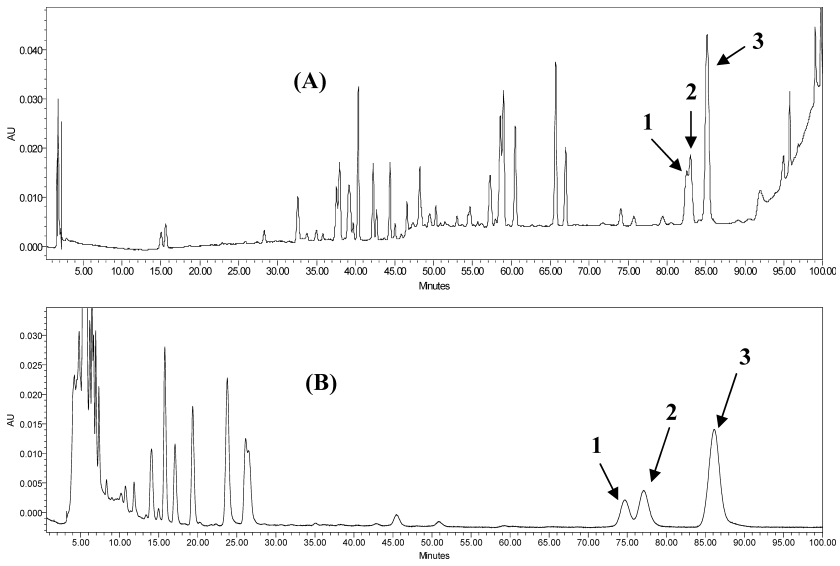

Fig. 2. Represented Chromatograms of Red Ginseng Products

(A) General ginsenosides isolation method (gradient elution from 20 to 90% for 100 min with acetonitrile, 1.6 ml/min, discovery C-18 column). (B) Selective isolation method (45% acetonitrile, 1.2 ml/min, 2×discovery C-18 column) for ginsenosides Rz_1 (**1**), Rk_1 (**2**) and Rg_5 (**3**).

Table 2. Content of Ginsenosides Rz_1 (**1**), Rk_1 (**2**), and Rg_5 (**3**)

Samples[a)]	Components and contents (%)		
	1	**2**	**3**
Prod.-1	0.03±0.00	0.06±0.01	0.24±0.01
Prod.-2	0.02±0.00	0.04±0.00	0.17±0.01
Prod.-3	0.11±0.02	0.18±0.01	0.58±0.03
Prod.-4	0.20±0.02	0.32±0.01	1.00±0.08
Prod.-5	0.11±0.01	0.20±0.01	0.58±0.04
Prod.-6	0.06±0.01	0.10±0.01	0.35±0.04
Prod.-7	0.08±0.01	0.12±0.01	0.40±0.05
Prod.-8	0.11±0.01	0.18±0.01	0.55±0.04
Prod.-9	0.15±0.02	0.26±0.01	0.76±0.04
Prod.-10	0.12±0.01	0.19±0.02	0.59±0.03
Prod.-11	0.14±0.01	0.24±0.02	0.69±0.02

The mean value of content (%) was resulted against sample weight and S.D. (n=3) of all data were given less then 0.002%. *a*) Each samples was the commercial product of Korea companies purchased from the Kungdong mart, Seoul, Korea, as water or alcohol extracts of red ginseng root.

0.172%), and from 0.17 to 0.69% (mean 0.537%), respectively (Table 2). The authors believe that the described method can be used to determine the levels of these three isomers in red ginseng products.

Experimental

General Experimental Procedures Optical rotations were measured with a JASCO DIP-370 digital polarimeter. FT-IR spectrum was obtained on a JASCO-40 spectrometer. The NMR spectra were taken on Varian UNITY 300 and JEOL JNM-A600 spectrometer. ESI-MS was obtained on a Fisions VG Quattro 400 mass spectrometer. Column chromatography was performed over Si gel 60 (Merk, 70/230 mesh). Thin layer chromatography (TLC) was carried out on pre-coated Silica-gel 60 F_{254} plates (0.25 mm, Merck), RP-18 F_{254} plate (0.25, Merck) and spots were detected under a UV light and by spraying 10% H_2SO_4 followed by heating. The HPLC system for isolation used Waters 2695 and 996 PDA detector and the column used reversed phase Si-column (Supelco C-18, 2.0×100 mm, 5 μm).

Plant Material The red ginseng roots for isolation were kindly supported from Korea Ginseng Corporation, and the several products of red ginseng extracts for quantitative analysis were purchased from the Kyungdong mart, Seoul, Korea.

Extraction and Isolation Fine powdered red ginseng root (1 kg), by steaming fresh *P. ginseng* roots at 95 °C for 2 h and then drying, was extracted with methanol refluxed for 24 h, and concentrated *in vacuo* to give a brown extract (100 g). The methanol extract was suspended in H_2O (2 l) and extracted with water saturated butanol (2 l×3). The concentrated butanol fraction (80 g) was suspended with excess water and then subjected to column chromatography on RP-18 (40—63 μm, 100 g). The column was eluted using a gradient aqueous acetonitrile (50→100%) to give three isomers (**1**, **2**, **3**) rich fraction. The isomers rich fraction (5 g) was subjected to recycling preparative liquid chromatography on C-18 column (Xtera prep C-18 5 μm, 30×100 mm with acetonitrile/water (52.5/47.5, 15 ml/min, seven recycled) to give **1** (20 mg), **2** (23 mg), and **3** (30 mg).

Ginsenoside Rz_1 (**1**): White amorphous powder; mp 188—191 °C; $[\alpha]_D$ −10.5° (c=0.2, MeOH); IR (KBr) ν_{max}: 3400, 2842, 1640 cm^{-1}; ESI-MS m/z 767 $[M+H]^+$; HR-ESI-MS m/z 767.4908 $[M+H]^+$; ^1H- and ^{13}C-NMR data: see Table 1.

HPLC Analysis of Compounds 1—3 Purchased red ginseng extracts (S-1—S-11, each 3 g) were dissolved in water (50 ml) then filtered with membrane filter (0.24 μm) for sample solutions. The HPLC separation for quantitative analysis of three isomers (**1**—**3**) was performed using a coupled to each other reverse phase column (Supelco C-18, 2.0×100 mm, 5 μm). Elution was isocratic solvent system with acetonitrile–water (45 : 55). The flow rate was 1.2 ml/min, and 20 μl aliquots of samples were injected for analysis and UV detection was carried out at 203 nm. Stock solutions (1 mg/ml) of isolated standard compounds **1**, **2**, and **3** were prepared individually in methanol, and different concentrations (0.5, 0.1, 0.05, 1.0 μg/ml) of these were loaded onto an HPLC for the preparation of the calibration functions.

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