

College of Pharmacy and Research Institute of Pharmaceutical Sciences¹; Natural Products Research Institute², Seoul National University, Seoul, Korea

Inhibitory activity of ginsenosides isolated from processed ginseng on platelet aggregation

JIN GYUN LEE¹, YONG YOOK LEE¹, BO WU¹, SUN YOUNG KIM², YONG JAE LEE¹, HYE SOOK YUN-CHOI², JEONG HILL PARK¹

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Prof. Jeong Hill Park, College of Pharmacy, Seoul National University, Seoul 151-742, Korea
hillpark@snu.ac.kr

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Seven ginsenosides, namely Rg₆ (1), F₄ (2), Rk₃ (3), Rh₄ (4), Rs₃ (5), Rs₄ (6) and Rs₅ (7) isolated from processed ginseng were evaluated for their effects on platelet aggregation induced by adenosine diphosphate (ADP), collagen, arachidonic acid (AA) and U46619 (thromboxane A₂ mimetic drug). Ginsenosides Rg₆, F₄ and Rk₃ showed inhibitory activity (IC₅₀ = 76 μM, 114 μM and 128 μM, respectively) on AA-induced platelet aggregation. The corresponding IC₅₀ values were comparable to that of acetylsalicylic acid (ASA) (63 μM). Compared to ASA (IC₅₀ = 468 μM) ginsenosides Rg₆, F₄, Rk₃ and Rh₄ were found to be more inhibitive (IC₅₀ = 286 μM, 87 μM, 187 μM and 119 μM, respectively) against U46619-induced aggregation. On the other hand, most of the ginsenosides (Rg₆, F₄, Rh₄, Rs₃, Rs₅) showed negligible effects on ADP and collagen-induced platelet aggregation. The acetylated ginsenosides (Rs₃, Rs₄ and Rs₅) had only mild effects on aggregation induced by four stimulators.

1. Introduction

Root of *Panax ginseng* C. A. Meyer (Araliaceae) is one of the most widely used traditional medicines in the Oriental countries. There are two typical traditional preparations; white ginseng and red ginseng. Because of the long history of ethnopharmacological evidence, red ginseng, usually produced by steaming raw ginseng, has been believed to provide enhanced bioactivities. Ginsenosides, regarded as the unique constituents of ginseng, are known to undergo structural changes on steaming. During heat processing of ginseng, several unique ginsenosides are produced mainly through deglycosylation of the dammarane glycoside backbone. Elevating the steaming temperature from 100 °C to 120 °C resulted in increased contents of less polar ginsenosides such as F₄, Rg₃, Rg₅, Rg₆, Rk₁, Rk₂, Rk₃, Rs₃, Rs₄ and Rs₅ through deglycosylation and dehydration at C₂₀ in dammarane backbone (Kwon et al. 2001). Processed ginseng produced by steaming at elevated temperature and pressure was reported to enhance the pharmacological activities, such as cytotoxic activity on various cancer cell types, radical scavenging effect and vasorelaxation activity. These enhanced activities of processed ginseng were attributed to the increased production of less-polar ginsenosides (Kim et al. 2000; Park et al. 2002c).

Ginseng has been well-known to have antithrombotic effects, and great efforts were made to elucidate the antithrombotic effects of ginseng and its components (Kuo et al. 1990; Lee et al. 1997; Matsuda et al. 1986; Teng et al. 1989; Yu et al. 2006). Ginsenosides Rg₂ and Rg₃ isolated from red ginseng were described as potent as acetylsalicylic acid (ASA) to inhibit antiplatelet aggregation (Lee et al. 1997; Matsuda et al. 1986), other ginsenosides showed only mild or negligible effects on platelet aggregation. Recently, representative saponins from processed ginseng, such as ginsenoside Rk₁ and Rg₅ were revealed to have potent antiplatelet aggregatory activity in the previous

study reported by our group (Lee et al. 2009). In addition to ginsenoside Rk₁ and Rg₅, processed ginseng contains various unique pharmacological active saponins which are less-polar than those of white ginseng (Kim et al. 1999a, b). However, antithrombotic effects of less polar ginsenosides from processed ginseng have not been fully evaluated yet. In the present work, seven unique ginsenosides isolated from processed ginseng were examined for their inhibitory activity on rat platelet aggregation.

2. Investigations, results and discussion

Seven ginsenosides isolated from processed ginseng were examined for their platelet anti-aggregatory activity. ASA, one of the most widely used anti-platelet drugs, was used as a positive control and the inhibitory effects of the isolated ginsenosides were compared with those of ASA. While most of the ginsenosides showed dose-dependent inhibitory activity against collagen, AA and U46619-induced platelet aggregation, all of them including ASA exhibited only negligible effect on ADP-induced platelet aggregation (data not shown). As shown in the Table, ginsenosides Rk₃ and Rs₄ were more inhibitive toward collagen-induced aggregation than ASA. Ginsenoside Rg₆ was as equivalently active as ASA on AA-induced aggregation, and showed mild inhibitive effect on U46619-induced aggregation. While ginsenosides F₄, Rk₃ and Rh₄ exhibited relatively stronger platelet anti-aggregatory activity than ASA against U46619-induced aggregation, their inhibitory effect on AA-induced platelet aggregation was observed to be lower than that of ASA. Ginsenoside F₄ exhibited the strongest inhibitive effect among seven tested compounds on U46619-induced platelet aggregation. It showed more than five-fold higher inhibitory potency than ASA. Ginsenosides Rk₃ and Rh₄ were found

to be 2.5 and 3.9 times more potent than ASA, in terms of inhibiting U46619-induced platelet aggregation. Dehydration of the protopanaxatriol (PPT) skeleton at C₂₀ position provides either exo-double bond ($\Delta^{20,21}$) or endo double bond ($\Delta^{20,22}$). Ginsenosides Rg₆ and Rk₃ were more inhibitive than their C₂₀-endo analogues against AA-induced aggregation. However, ginsenosides F₄ and Rh₄ showed stronger inhibitory effect on U46619-induced aggregation than their C₂₀-exo analogues. The result suggests that the C₂₀-exo double bond is more favorable for inhibiting the formation of TXA₂, and C₂₀-endo double bond is more favorable as TXA₂ receptor antagonists. Meanwhile, ginsenosides Rs₃, Rs₄ and Rs₅, which have acetylated glycosyl moiety at C₃ of dammarane backbone showed only very mild effects on platelet aggregation induced by all four stimulants used in this work. Ginsenosides Rs₄ and Rs₅ showed very mild inhibitory activity on collagen or U46619-induced platelet aggregation.

In summary, seven ginsenosides isolated from processed ginseng were evaluated for their platelet anti-aggregating effect. Ginsenosides F₄ and Rh₄, which have $\Delta^{20,22}$ double bond, showed stronger inhibitory effect on U46619-induced platelet aggregation than their homologues which have a $\Delta^{20,21}$ double bond. Ginsenosides Rg₆, F₄ and Rk₃ showed mild inhibitory effect on AA-induced aggregation.

3. Experimental

3.1. General

The HPLC system consisted of two L-7100 pumps (Hitachi, Japan) coupled with a L-4000 UV detector (Hitachi, Japan) and a Sedex evaporative light scattering detector (ELSD, Sedere, France). Phenomenex® Gemini C₁₈ column (5 μ m, 250 $\times$ 10 mm) and Phenomenex® Gemini C₁₈ column (5 μ m, 250 $\times$ 4.6 mm) were used for isolation and purity assessment of the compounds, respectively. NMR (¹H and ¹³C) and MS spectra of the isolated compounds were recorded using an Avance 500 Spectrometer (Bruker, Germany) and a JMS-700 Mass spectrometer (Jeol, Japan), respectively. The Number of platelets was measured on an Excell 18 MWI platelet counter (DANAM Electronics, USA) and the level of platelet aggregation was determined using an 490-X optical aggregometer (Chrono-Log Corp., USA).

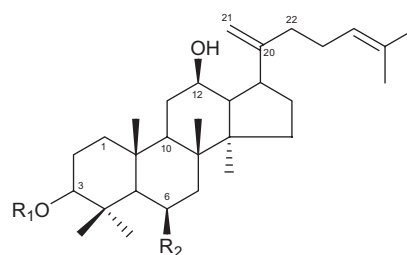
Male Sprague-Dawley rats (250 $\pm$ 20 g) were purchased from Orient-Bio (Korea) and the experiment was conducted in accordance with the Guide for the Care and Use of Laboratory Animals by Seoul National University. Adenosine 5'-diphosphate dicyclohexylammonium salt (ADP), sodium arachidonate (AA), U46619 (9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin F₂ α) and acetylsalicylic acid (ASA) were purchased from Sigma Aldrich Chem. Co. (St. Louis, MO, USA). Collagen was purchased from Chrono-Log Co. (Havertown, PA, USA). Unless stated otherwise, all other reagents were from Sigma Aldrich. All the chemicals, reagents, or solvents used were of analytical grade or cell culture tested.

3.2. Plant material

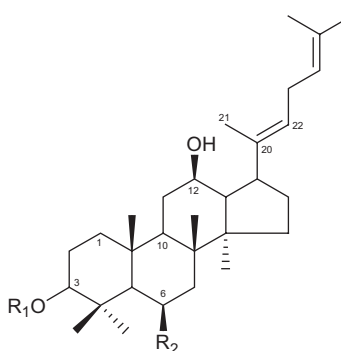
White ginseng (dried root of *Panax ginseng* C. A. Meyer, 4 years old) was purchased from an oriental medicine market in Keumsan, South Korea. White ginseng was steamed at 120 °C for 3 h in an autoclave as described previously (Kwon et al. 2001). The voucher specimens of the white ginseng (ANALAB-0701) and the processed ginseng (ANALAB-0702) were deposited in the herbarium of the College of Pharmacy, Seoul National University.

3.3. Extraction and isolation

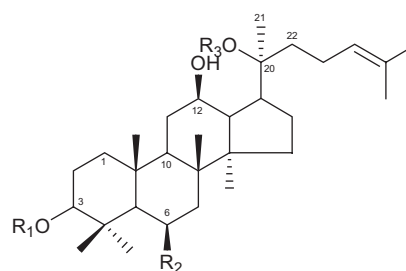
The processed ginseng (2 kg) was extracted thrice with methanol (MeOH) (3 l) and after solvent evaporation, the residue (380 g) was suspended in H₂O (5 l) and extracted with *n*-butanol (*n*-BuOH, 5 l). A portion of evaporated *n*-BuOH soluble fraction (30 g) was subjected to silica-gel column chromatography eluting with a CHCl₃-MeOH stepwise gradient (40:1 $\rightarrow$ 10:1) yielding 10 fractions (Fr. 1–Fr. 10). Fr. 5, Fr. 6 and Fr. 8, dissolved in MeOH, were subjected to semi-preparative HPLC/UV and eluted isocratically with aqueous acetonitrile (50% for Fr. 5, 65% for fr. 6, 60% for fr. 8). Each peak on the chromatogram was collected repeatedly. Seven ginsenosides were isolated; Rg₆ (16.5 mg; from Fr. 5), F₄ (18.7 mg; from Fr. 5), Rk₃



Ginsenoside	R ₁	R ₂
Rg ₆	H	OGlc-Rha
Rk ₃	H	OGlc
Rs ₅	Glc-Glc-Ac	H



Ginsenoside	R ₁	R ₂
F ₄	H	OGlc-Rha
Rh ₄	H	OGlc
Rs ₄	Glc-Glc-Ac	H



Ginsenoside	R ₁	R ₂	R ₃
Rs ₃	Glc-Glc-Ac	H	H

Glc: -D-glucopyranosyl, Rha: -L-rhamnopyranosyl, Ac: 6'-O-acetyl

(17.1 mg; from Fr. 5), Rh₄ (19.2 mg; from Fr. 5), Rs₃ (13.6 mg; from Fr. 8), Rs₄ (15.6 mg; from Fr. 6), Rs₅ (16.3 mg; from Fr. 6). The structures of the isolated compounds were elucidated by direct comparison of their ¹H-, ¹³C- NMR and MS spectra including HPLC retention times with those of authentic samples isolated by our group (Park et al. 2002a,b). The purity of the isolated compounds, as analyzed by HPLC, were >95%.

3.4. Assay for antiplatelet-aggregatory activity

Whole blood was collected from the heart of male SD rat using a syringe containing 2.2% aqueous solution of sodium citrate. Supernatant platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 200 $\times$ g for

Table: Platelet anti-aggregatory activity of ginsenosides isolated from processed ginseng

Ginsenoside	IC ₅₀ (μM)		
	Collagen ^a	AA ^{b,d}	U46619 ^{c,e}
Rg ₆	>700	76	286
F ₄	>700	114	87
Rk ₃	192	128	187
Rh ₄	>700	>200	119
Rs ₃	>700	>200	>700
Rs ₄	302	>200	352
Rs ₅	>700	>200	421
ASA ^f	586	63	468

^a collagen (3–4 μg/ml),^b AA (40–50 μM),^c U46619 (3–4 μM),^d ^e in presence of the near-threshold concentration of collagen ^d (1.0–1.4 μg/ml) and ^e (0.8–1.2 μg/ml),^f ASA: acetylsalicylic acid which was used as a positive control

10 min. The residue was centrifuged at 1500 × g for 15 min to obtain the platelet poor plasma (PPP). Then, the platelet count was adjusted to 4.0–4.5 × 10⁸/ml by diluting PRP with PPP. Antiplatelet aggregatory activity was measured by the turbidimetric method described by Born and Cross (1963). An aliquot (500 μl) of platelet count adjusted PRP was preincubated for 3 min, and the test sample (or vehicle) was added. After 30 s, an aggregation inducing agent was added. Because of poor solubility of isolated compounds in saline, DMSO (final concentration 1.0%) was used as a vehicle. AA and U46619-induced aggregation was determined in presence of a threshold concentration of collagen that induces only change in platelet shape but no aggregation (Pyo, Lee and Yun-Choi 2002). Percent inhibition was calculated as follows:

Inhibition (%)

$$= \left(1 - \frac{\text{Maximal aggregation of sample-treated aPRP}}{\text{Maximal aggregation of vehicle-treated aPRP}} \right) \times 100$$

All experiments were performed in triplicate. IC₅₀ values were calculated from the linear regression of the plot of mean values (n=3) of percent inhibition *versus* concentration of corresponding ginsenoside. Sigma Plot software was used for graphing. Regression equations were calculated using Regression Wizard from the Sigma Plot equation library.

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