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Stereospecificity in hydroxyl radical scavenging activities of four ginsenosides produced by heat processing

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Abstract—The activity-guided fractionation of sun ginseng (SG, heat processed *Panax ginseng* C. A. Meyer at 120 °C) was carried out to identify its main active hydroxyl radical (·OH) scavenging components. As a result, the *n*-BuOH fraction mainly consisting of ginsenosides showed the strongest activity. Of several ginsenosides of SG, the ·OH scavenging activities of relatively high contents of 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 were compared. Rg_5 and 20(*S*)-Rg_3 showed strong ·OH scavenging IC_{50} values of 0.15 and 0.44 mM, respectively, and these activities were prominently higher than each of their respective isomers. Therefore, stereospecificity exists in the ·OH scavenging activities of ginsenosides produced by heat processing. Especially, the double bond at carbon-20(22) or the OH group at carbon-20 geometrically close to OH at carbon-12 is thought to increase the ·OH scavenging activity of ginsenosides.

Traditionally, the root of ginseng (*Panax ginseng* C. A. Meyer, Araliaceae) has been processed to make white ginseng (WG, roots air-dried after peeling) and red ginseng (RG, roots steamed at 98–100 °C without peeling) to enhance its preservation and efficacy. RG is more common as an herbal medicine than WG in Asian countries, because steaming induces changes in the chemical constituents and enhances the biological activities of ginseng.[1–3] Recently, a method to increase the content of RG specific ginsenosides such as Rg_3, Rk_1, and Rg_5 by steaming WG at a higher temperature than RG was developed. This novel heat processed ginseng is termed sun ginseng (SG), and we have been investigating its enhanced free radical scavenging activity compared to conventional ginsengs and its active constituents.[4–6]

The hydroxyl radical (·OH) is an extremely reactive and short-lived species that can attack biological molecules such as DNA, proteins, and lipids. The reactivity of ·OH has been related to several human diseases such as neurodegenerative disease and diabetes. Therefore, its scavenging activity has received much attention.[7–9] Especially, in our previous research, SG showed an improved ·OH scavenging activity compared to WG and RG,[5] and so we needed to clarify the main active ·OH scavenging components of SG. Therefore, in the present study, the activity-guided fractionation of SG was carried out. To test the ·OH scavenging activity, an electron spin resonance (ESR) spectrometer was used because it is widely employed for detecting ·OH, and even samples with a low solubility such as ginsenosides are suitable for ESR measurement.[10] In addition, (−)-epigallocatechin 3-*O*-gallate (EGCg) was used as an ·OH scavenging positive control.[11]

Ginseng extract is known to scavenge free radicals such as 1,1-diphenyl-2-picrylhydrazyl, carbon-centered radical, superoxide, nitric oxide, peroxynitrite, and ·OH. In addition, these scavenging activities are improved by heat processing.[5,6,9,12] Especially, an extremely reactive ·OH radical was effectively scavenged by SG compared to WG or RG in our previous research,[5] and therefore we needed to clarify the main active components of SG. Thus, the activity-guided fractionation of SG extract was carried out. First, SG was separated into its 90% water-soluble fraction (mainly containing polysaccharides) and residual 10% MeOH eluate (containing ginsenosides, phenolic acids, etc.) by HP 20 resin.[13] In the ·OH scavenging activity tests of SG and its two fractions, SG inhibited ·OH generation to 70%, and the lower content of MeOH eluate (inhibited to 55%) showed a stronger activity than the water eluate (inhibited to 86%) at the concentration of 1 mg/mL (Fig. 1). Therefore, the MeOH eluate was thought to contain the active compo-

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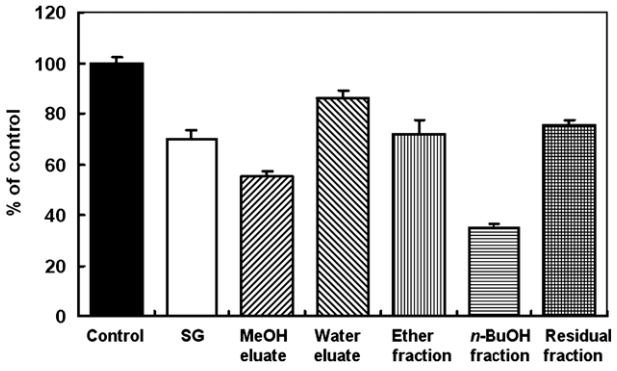


Figure 1. The ·OH scavenging activities of SG[24] and its fractions[25] at 1 mg/mL.

nents and was further separated into three fractions of ether, *n*-BuOH, and residual fractions by the reported method.[14] Of these, the *n*-BuOH fraction containing mainly ginsenosides showed the strongest activity and the next were in the order of ether and residual water fractions (Fig. 1). The *n*-BuOH fraction was thought to contain the main active components because it occupied about 70% of the MeOH eluate and showed the strongest inhibition of ·OH generation to nearly 35%. Therefore, the *n*-BuOH fraction was analyzed by HPLC (Fig. 2) and relatively high contents of four ginsenosides, 20(*S*)-Rg_3 32.8%, 20(*R*)-Rg_3 7.3%, Rk_1 15.7%, and Rg_5 18.6%, were detected, isolated, and these ginsenosides are known as the major ginsenoside constituents of SG.[4,15,16] Especially, 20(*R*)-Rg_3 and 20(*S*)-Rg_3 are epimers of each other depending on the position of the hydroxyl (OH) group on carbon-20 (Fig. 3), and this epimerization is known to be produced by the selective attack of the OH group after elimination of the glycosyl residue at carbon-20 during the steaming process.[17,18] Similarly, in the case of Rk_1 and Rg_5, these ginsenosides are positional isomers of each other depending on the position of the double bonds on carbon-20(21) or -20(22), as shown in Figure 3, and this double bond is known to be produced by the elimination of H_2O at carbon-20 of Rg_3 by high pressure and temperature.[4]

For the four isolated ginsenosides, ·OH scavenging activity tests were carried out and they showed characteristic activity and structure relationships (Fig. 4). Rg_5 showed the strongest activity and the next were in the order of 20(*S*)-Rg_3, Rk_1, and 20(*R*)-Rg_3 at a concentration of 2 mM. Rg_5 and 20(*S*)-Rg_3 showed a stronger activity than that of the positive control, EGCg, at the same concentration (2 mM). In a comparison of the epimers of Rg_3, 20(*S*)-Rg_3 strongly inhibited ·OH generation to 20%, but 20(*R*)-Rg_3 showed a lower value of 72%. Similarly, Rg_5 (inhibited to 16%) showed a stronger activity than Rk_1 (inhibited to 68%). Therefore, stereospecificity exists in the ·OH scavenging activity of ginsenosides. Especially, the double bond at carbon-20(22) or the OH group at carbon-20 geometrically close to OH at carbon-12 is thought to increase the ·OH scavenging activity of ginsenoside (Figs. 3 and 4). In addition, stereospecificity in the medicinal efficacy of these ginsenosides was reported by several researchers. The OH group of 20(*S*)-Rg_3 is better aligned with the OH acceptor group in the ion channels than that of 20(*R*)-Rg_3, and is important for Na^+ channel regulation.[19,20] Moreover, 20(*S*)-Rg_3 is reported to provide neuroprotection against cerebral ischemia-induced injury in the rat brain through reducing lipid peroxides and scavenging free radicals.[21] Similarly, Rg_5, not Rk_1, is known as one of the anticarcinogenic compounds of RG.[22]

On the other hand, the strong ·OH scavenging activities of Rg_5 and 20(*S*)-Rg_3 were further compared at the concentrations of 0.2 and 1.0 mM for the calculation of their IC_{50} values. These two ginsenosides strongly inhibited DMPO-OH signal generations (Fig. 5), graphically represented in Figure 6. The IC_{50} values of Rg_5 and 20(*S*)-Rg_3 were 0.15 and 0.44 mM, respectively. In addition, the IC_{50} value of EGCg, the ·OH scavenging positive control, was 3.76 mM (data not shown). Therefore, the ·OH scavenging activity of SG is thought to be closely related to these two ginsenosides produced by heat processing. Although we clarified two strong ·OH scavenging ginsenosides, it was difficult to assess whether these ginsenosides were simply good metal chelators

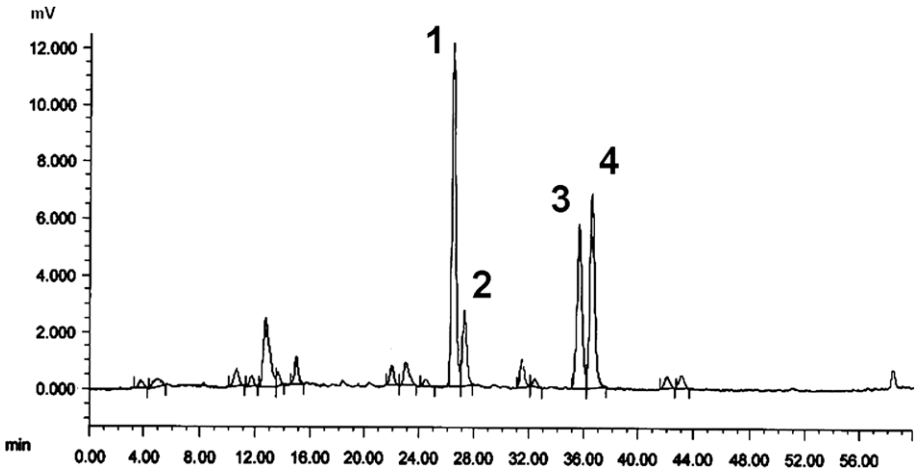
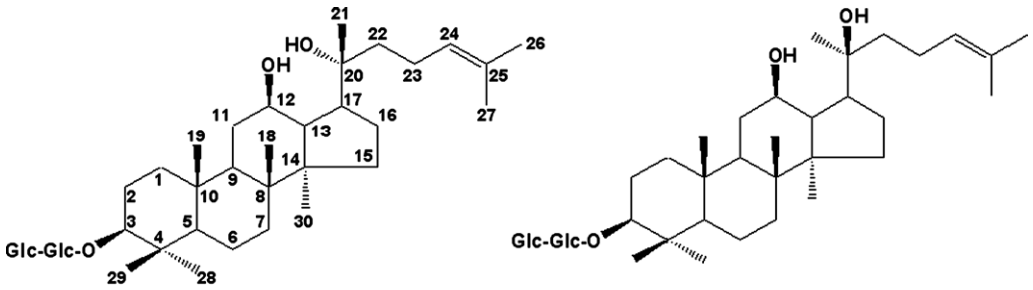


Figure 2. HPLC chromatogram of the *n*-BuOH fraction[26]: (1) 20(*S*)-Rg_3; (2) 20(*R*)-Rg_3; (3) Rk_1; (4) Rg_5.



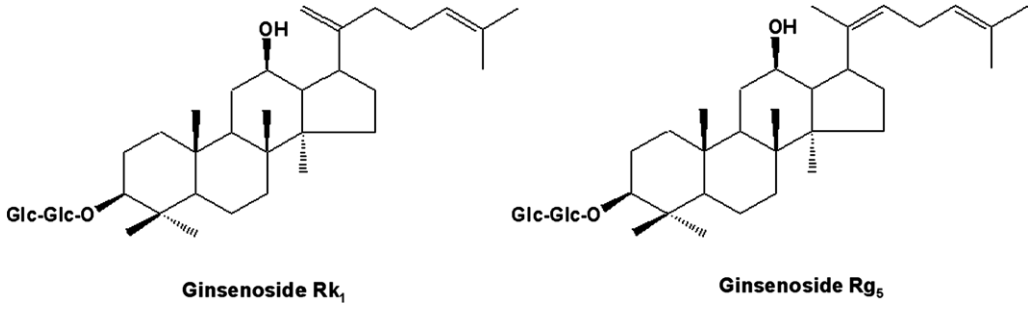


Figure 3. Structures of ginsenosides[27]: -Glc, D-glucopyranosyl.

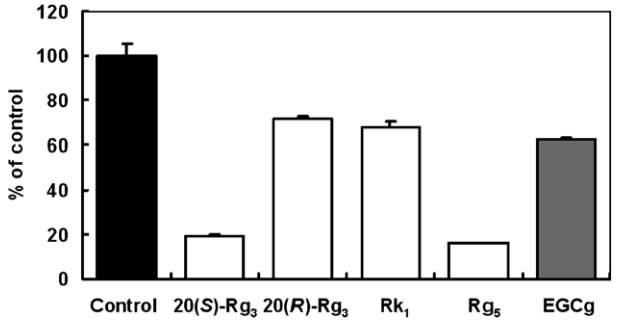


Figure 4. The ·OH scavenging activities of four ginsenosides at 2 mM.

on Fenton reaction or direct ·OH scavengers. However, many antioxidants are also metal chelators, and the direct scavenging of ·OH by dietary antioxidants in a biological system is known to be unrealistic because the cellular concentration of dietary antioxidants is negligible compared with other biological molecules. Thus, the ·OH scavenging capacity is mainly due to their metal chelating capability, and metal chelators may act as antioxidants.[23] On the other hand, four ginsenosides used in this study showed no or weak free radical, nitric oxide, scavenging activities in our previous study,[6] and there are few reports describing the direct free radical scavenging activities of ginsenosides, even with the long history of ginseng research. Therefore, we suggest that the ·OH scavenging activities of ginsenosides measured by ESR are mainly due to their metal chelating capability. As there are still unsolved issues about the ·OH scavenging activities of minor ginsenosides of SG and the need to investigate active components in other fractions, we want to clarify these subjects in a future study.

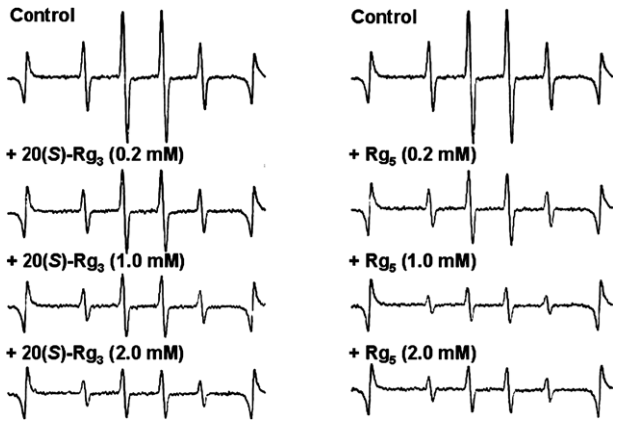


Figure 5. ESR spectra of DMPO-OH adducts formed by the Fenton reaction[28] with 20(*S*)-ginsenoside Rg_3 and ginsenoside Rg_5.

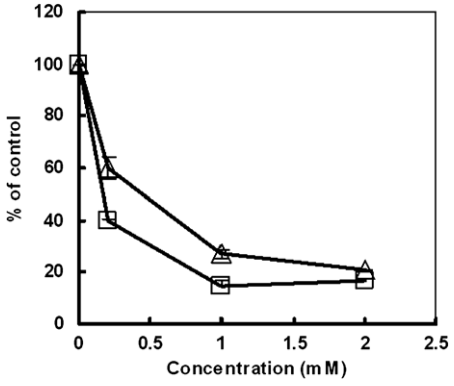


Figure 6. Comparison of the ·OH scavenging activities of 20(*S*)-Rg_3 (△) and Rg_5 (□).

In summary, we first reported the ·OH scavenging activities of ginsenoside-Rg_5 and 20(*S*)-Rg_3 by the use of ESR. In addition, these two ginsenosides showed a stronger activity than each of their respective isomers. Therefore, there is stereospecificity in the ·OH scavenging activities of ginsenosides produced by heat processing. Especially, the double bond at carbon-20(22) or the OH group at carbon-20 geometrically close to OH at carbon-12 is thought to increase the ·OH scavenging activity of ginsenosides. These structural characteristics of ginsenosides to scavenge ·OH could be useful to develop novel antioxidant.

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24. SG was made by steaming WG at 120 °C and dried at 50 °C for 3 days as previously described in *J. Nat. Prod.* **2000**, *63*, 1702. WG was purchased from a local ginseng market in Seoul (Korea).
25. Several fractions of SG used in this study were prepared by the following procedures. SG MeOH extract (30 g) was suspended in water and then the water-soluble polysaccharide fraction was separated by Diaion HP 20 (Mitsubishi Chemical, Tokyo, Japan) column chromatography using water as an eluting solution followed by elution with MeOH. Each solution was evaporated in vacuo to give the water eluate (27 g) and the MeOH eluate (3 g). The MeOH eluate was re-suspended in water and further partitioned into ether and aqueous layers, and the aqueous layer was then extracted with *n*-BuOH saturated with water for the preparation of the total ginsenosides fraction. Each fraction was dried under reduced pressure and the ether (800 mg), *n*-BuOH (2.1 g), and residual fractions (100 mg) were obtained.
26. Ginsenosides in the *n*-BuOH fraction were analyzed by HPLC. A Hitachi (Tokyo, Japan) L-7100 liquid chromatograph fitted with a C-18, reverse-phase column (5 μm, 25 cm × 4 mm I.D.; YMC-Pack Pro) utilizing the solvent gradient system was used. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), and the flow rate was 1 mL/min. The detector was a SEDEX 55 ELSD (Sedere, France). The gradient elution was used as follows: 0 min, 15% B; 10 min, 34.5% B; 25 min, 47.5% B; 40 min, 80% B; and 50 min, 100% B. Ginsenosides were identified by co-injection with standard ginsenosides.
27. For the isolation of 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5, the *n*-BuOH fraction of SG was applied to a silica gel column eluting with $CHCl_3$–MeOH (30:1 → 20:1 → 10:1 → 1:1) to afford subfractions, and it was further purified with preparative HPLC with a detection wavelength of 203 nm. The structures of ginsenosides were confirmed by comparing the chemical shifts in ^{13}C NMR and ^{1}H NMR with standard samples.
28. The ESR spectra were recorded on a JES-TE100 ESR spectrometer (JEOL, Tokyo, Japan). The experimental parameters were as follows: temperature, ambient; microwave power, 1.02 mW; modulation frequency, 100 kHz; modulation width, 0.16 mT; sweep width, 5.0 mT; sweep time, 0.5 min; center field, 339.550 mT; time constant, 0.03 s; and receiver gain, 1. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was used as a spin-trapping reagent for ·OH. Mn^{2+} was used as an external standard to calculate the relative amounts from the ESR signal intensity. Twenty microliters of DMPO (1/10 diluted with H_2O, v/v) were mixed with 38 μL of 0.2 mM $FeSO_4{\cdot}7H_2O$ and 37 μL of 1 mM diethylenetriaminepentaacetic acid. The mixture was stirred with 30 μL of sample solution and 75 μL of 1 mM H_2O_2. The solutions were transferred to a capillary tube and placed in the cavity of the ESR spectrometer for measurement. After 5 min, the ESR signal was taken to measure the level of inhibition of ·OH by the samples. Measurements were repeated three times for each sample. The inhibition of ·OH was determined by the ratio of the peak height of the DMPO-OH spin adduct to the signal of Mn^{2+} and compared to the ratio of the control.