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The chemical and hydroxyl radical scavenging activity changes of ginsenoside-Rb_1 by heat processing

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ABSTRACT

The chemical and hydroxyl radical (.OH) scavenging activity changes of ginsenoside Rb_1 (Rb_1) by heat processing were investigated in this study. Rb_1 was changed into 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 by heat processing through glucosyl elimination and epimerization of carbon-20 by SN1 reaction. The glucosyl moiety, separated from Rb_1, made Maillard reaction product (MRPs) with glycine. The generations of 20(S)-Rg_3 and MRPs were related to the increased .OH scavenging activity of Rb_1 by heat processing.

The root of ginseng, *Panax ginseng* C. A. Meyer (Araliaceae), has been heat processed to improve its medicinal efficacies in Korea. Steaming process is known to induce a structural change of ginsenoside and to enhance the biological activities of ginseng.[1–4] Although there are several reports about the heat processing or intestinal bacteria-induced chemical and biological activity changes of ginsenosides, the interaction of ginsenoside with amino acids during heat processing was not fully elucidated yet.[5,6] Therefore, we have been investigating the chemical and antioxidant activity changes of ginsenosides by Maillard reaction.

Ginsenoside-Rb_1 (Rb_1) is a well-known diol-type triterpene glycoside that exists most abundantly in *Panax ginseng*, and is known to have anti-inflammatory and antioxidant effects.[7–9] Rb_1 rescued hippocampal neurons from lethal damage caused by the hydroxyl radical (.OH)-promoting agent $FeSO_4$ in vitro, and the Fenton reaction containing *p*-nitrosodimethylaniline confirmed the .OH inhibiting activity of Rb_1.[10] In addition, the .OH scavenging activity test using electron spin resonance spectrometer (ESR) was suggested to be the most appropriate to test the antioxidant activities of ginsenosides, and this effect was closely related to the ferrous metal ion-chelating activities of ginsenosides from our previous researches.[5,6,11]

On the other hand, most major Maillard reactions are represented by sugar-amino acid groups, and the reaction between reducing sugars and amino acid produces strong reducing materials such as amino reductants during heat treatment.[12,13] Rb_1 has glucosyl moiety at carbon-20, which can be easily separated by heat processing, and glycine is a frequently used amino acid in the Maillard reaction model system, and is also contained in *Panax ginseng*.[14,15] In addition, we already demonstrated the significant generation of Maillard reaction products (MRPs) from the heat processing model experiment using Rb_1 and glycine. However, arginine, major amino acid of ginseng, inhibited Maillard reaction of Rb_1 with unknown reason from our previous report.[6] Therefore, the chemical and .OH scavenging activity changes of Rb_1 by heat processing with glycine were investigated in this study before the further study on the other amino acids.

Rb_1 consists of four hydrophobic ring steroid-like structures with hydrophilic sugar moieties at carbon-3 and -20 (Fig. 1).[2,16] To elucidate the chemical and antioxidant activity changes of Rb_1

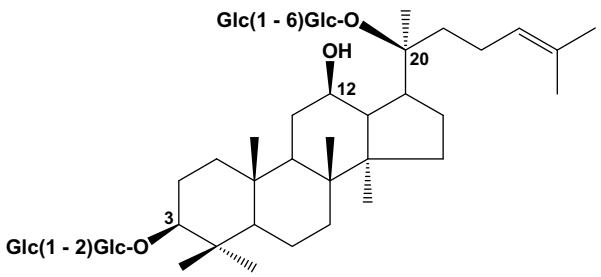

Figure 1. The chemical structure of ginsenoside Rb_1. –Glc, D-glucopyranosyl.

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by Maillard reaction, we investigated the Maillard reaction model experiment using Rb_1 and glycine,[17] and the .OH scavenging activity changes were tested with ESR.[18] Figure 2 shows the comparisons in .OH scavenging activities of Rb_1, heat processed Rb_1, Rb_1-glycine mixture, and heat processed Rb_1-glycine mixture. Rb_1 strongly inhibited .OH production to about 24% at the concentration of 0.5%, but the effect after heat processing was lowered to 42% (Fig. 2(A) and (B)). On the other hand, Rb_1-glycine mixture inhibited .OH production to about 50%, which effect was about the half of Rb_1, but the effect of heat processed Rb_1-glycine mixture had a stronger value of 37% at the concentration of 0.5%. In addition, there showed nearly no .OH scavenging activity in glycine (data not shown). Consequently, the .OH inhibiting activity of Rb_1 was decreased by heat processing, but it was increased when heat processed with glycine (Fig. 2(B) and (C)). Therefore, comparisons of the constituent analysis[19] and .OH scavenging activities of components produced by the heat processing of Rb_1-glycine mixture were carried out.

As shown in the HPLC chromatograms of Rb_1 and heat processed Rb_1, Rb_1 (1 mg) was detected at about 17 min when not steamed (Fig. 3(A)) and it disappeared, and the contents of 20(*S*)-Rg_3 (146 μg), 20(*R*)-Rg_3 (201 μg), Rk_1 (102 μg), and Rg_5 (110 μg)[20] were increased by heat processing (Fig. 3(B)). In addition, the increased peak area at about 2.3 min shown in Figure 3(B) was determined as glucose from the GC-MS analysis (data not shown). In the case of steaming model experiment using Rb_1-glycine mixture, the glycine and Rb_1 were detected at about 2.3 and 17.0 min, respectively, when not steamed (Fig. 3(C)). Then, all of the Rb_1 disappeared, and the contents of 20(*S*)-Rg_3 (196 μg), 20(*R*)-Rg_3 (167 μg), Rk_1 (102 μg), and Rg_5 (108 μg) were increased as shown by steaming of Rb_1, but the generated content of 20(*S*)-Rg_3 was higher than that of heat processed Rb_1 (Fig. 3(B) and (D)). On the other hand, the change in content of glycine was not confirmed because its peak was overlapped with glucose peak produced by steaming as shown in Fig. 3(D). 20(*S*)-ginsenosides and 20(*R*)-ginsenosides are epimers of each other depending on the geometrical position of the hydroxyl group (OH) on carbon-20. Especially, this epimerization is known to occur by the selective attack of the OH group after the elimination of the glycosyl residue at carbon-20 during the steaming process.[14,16] In addition, more less-polar ginsenosides such as Rk_1 and Rg_5 are known to be easily produced by the elimination of H_2O at carbon-20 of Rg_3 under high pressure and temperature conditions like in autoclaving.[3,5] Therefore, Rb_1 was gradually changed into 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 by heat processing, but the generated content of 20(*S*)-Rg_3 was higher than that of 20(*R*)-Rg_3 when Rb_1 was heat processed with glycine (Fig. 3(B) and (D)) as determined previously,[6] and there was a need to examine the .OH scavenging activities of these less-polar ginsenosides produced by heat processing for the identification of active components.

When the .OH scavenging activities of Rb_1, glycine, 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 were compared, Rb_1 inhibited .OH production to about 47% at the concentration of 0.05%, but glycine showed slight or no .OH scavenging activity (Fig. 4(A)). In the comparison of ginsenosides produced by steaming, 20(*S*)-Rg_3 and Rg_5 strongly inhibited .OH generation to about 16 and 22%, respectively, at the concentration of 0.05% (Fig. 4(A)), but the effects of 20(*R*)-Rg_3 and Rk_1 were comparably lower than that of Rb_1 (Fig. 4(A) and (B)). The stronger .OH inhibiting activities of 20(*S*)-Rg_3 and Rg_5 than their epimers were suggested to be mediated by geometrically closer double bond and OH group at carbon-20 to OH group at carbon-12.[11] Although the strongly .OH inhibiting ginsenosides such as 20(*S*)-Rg_3 and Rg_5 were generated by heat processing, it accompanied the increases in 20(*R*)-Rg_3 and

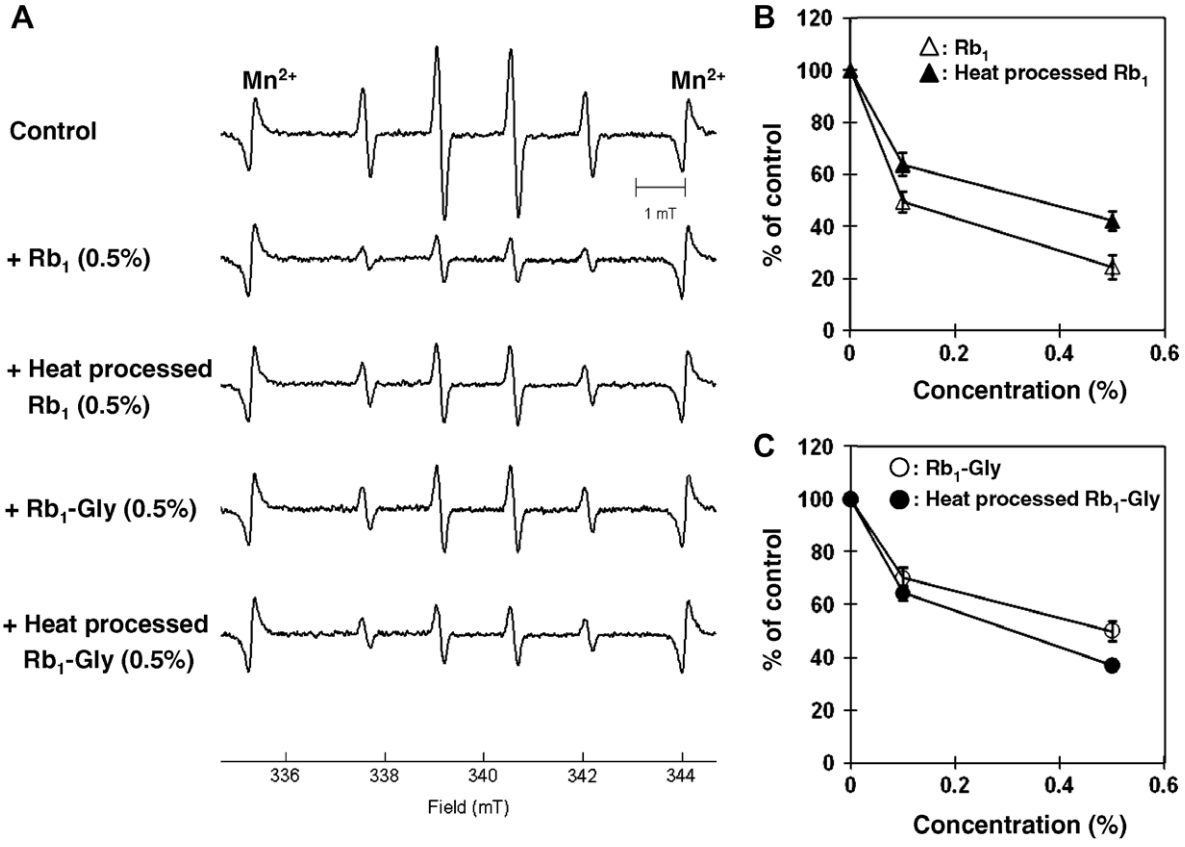

Figure 2. The comparison in the .OH scavenging activities of Rb_1, heat processed Rb_1, Rb_1-glycine mixture, and heat processed Rb_1-glycine mixture.

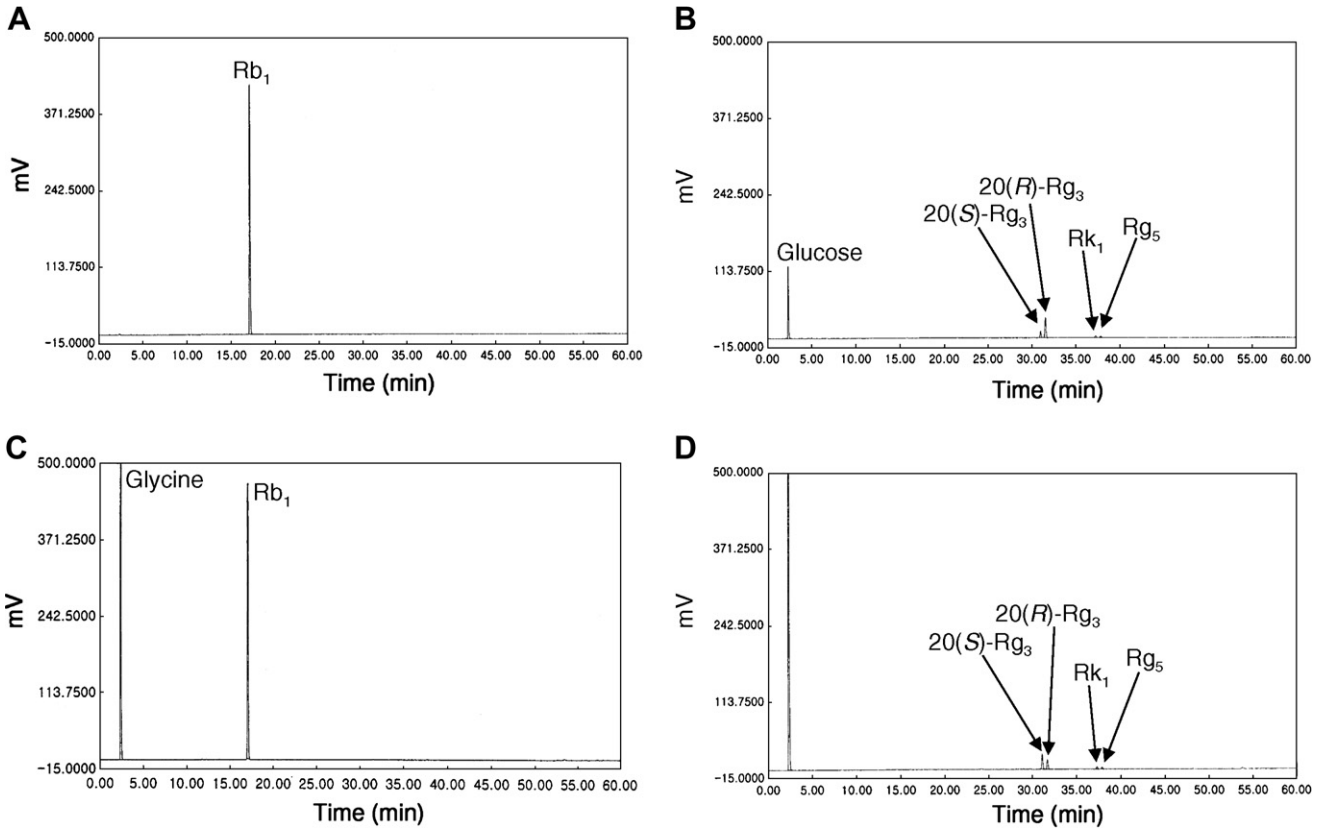

Figure 3. HPLC chromatograms of (A) Rb_1, (B) heat processed Rb_1, (C) Rb_1-glycine mixture, and (C) heat processed Rb_1-glycine mixture.

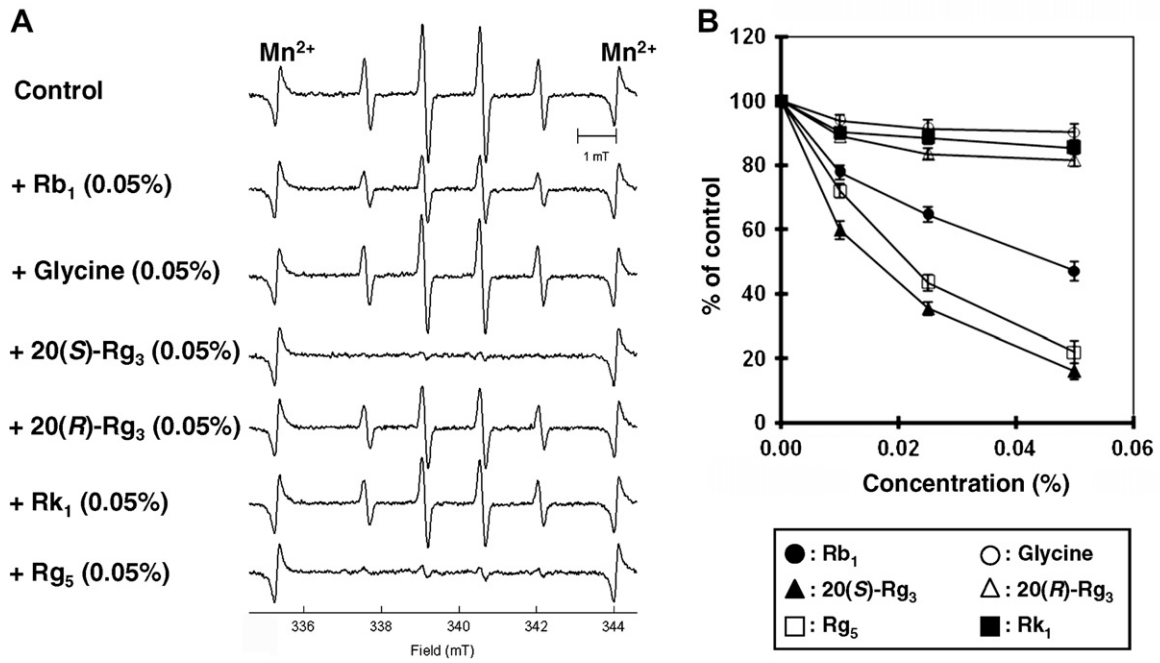

Figure 4. The comparison in the **.**OH scavenging activities of Rb_1, glycine, 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5.

Rk_1 weak **.**OH inhibitors. Therefore, the effect of these mixed ginsenosides was thought to be neutralized or lowered, and there showed no significant increase in the **.**OH inhibiting activity when the Rb_1 was heat processed. However, the generated content of 20(*S*)-Rg_3 was more higher than that of 20(*R*)-Rg_3 when Rb_1 was heat processed with glycine (Fig. 3(B) and (D)), and the increased **.**OH scavenging activity of Rb_1-glycine mixture by heat processing was thought to be partially mediated by effect of 20(*S*)-Rg_3. These results were similar with our previous report using Rb_2, another major ginsenoside, and glycine.[21]

We have confirmed that the MRPs may have little or no effects on the increased .OH scavenging activity of Rb_2-glycine mixture by heat processing in our previous report.[21] The novel point of this study is that the MRPs, which generated from Rb_1-glycine mixture by heat processing, have .OH scavenging activity. The development of color is known as an important and obvious feature of the Maillard reaction, and heat-induced antioxidants including melanoidins and reductones are known to be formed by this reaction.[13,22] Figure 5 shows the comparison in browning levels of un-treated Rb_1 and its steamed products at 120 °C with or without glycine. The absorbance values at 420 nm of un-treated Rb_1 and glycine were 0.0053 and 0.0002 A.U., respectively, and they were increased to 0.0073 and 0.0042 A.U. when heat processed at 120 °C for 3 h, respectively (Fig. 5). No significant changes in the browning compound levels were noted when Rb_1 and glycine were heat processed separately, but the browning level of heat processed Rb_1-glycine mixture was significantly high value of 0.3182 A.U. than the others (Fig. 5). Therefore, heat-induced antioxidants including melanoidins and reductones were thought to be generated by the Maillard reaction as mentioned above. Considering the HPLC data of un-treated and heat processed Rb_1-glycine mixture, Rb_1 was gradually changed into 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 by heat processing, and its sugar moieties at carbon-20 were separated (Fig. 3(B)). The separated sugar moieties such as glucose or maltose were thought to form MRPs with the added glycine, but we could not identify the molecular structures of MRPs in present study because their structures are extremely complex and largely unknown.[21] Then, the browning compound level and .OH scavenging activity changes of the MRPs produced from glucose–glycine and maltose–glycine mixtures were examined.

Figure 6 shows the changes in .OH scavenging activities and browning compound levels of MRPs generated from glucose–glycine and maltose–glycine mixtures. MRPs generated from glucose–glycine and maltose–glycine mixtures inhibited .OH generation to about 37% and 77%, respectively, at the concentration of 0.05% (Fig. 6 (A)). In addition, the browning compound levels at the concentration of 0.05% of MRPs generated from glucose–glycine and maltose–glycine mixtures were 1.019 and 0.117 A.U., respectively. These values were dose-dependently increased as shown in Fig. 6(B) and (C), and the brown color of MRPs generated from glucose–glycine mixture was stronger than that of maltose–glycine mixture. In addition, the .OH scavenging activity of glucose–glycine mixture was stronger than that of Rb_1 at the same concentration (Figs. 4 (B) and 6(A)). Therefore, it is clear that the MRPs generated from the separated glucosyl moiety of Rb_1 and glycine have .OH scavenging activity, and the changes in browning levels of ginsengs or ginsenosides by heat processing were thought to be related to the increase in .OH scavenging activity. On the other hand, the little or no effects of MRPs from Rb_2-glycine

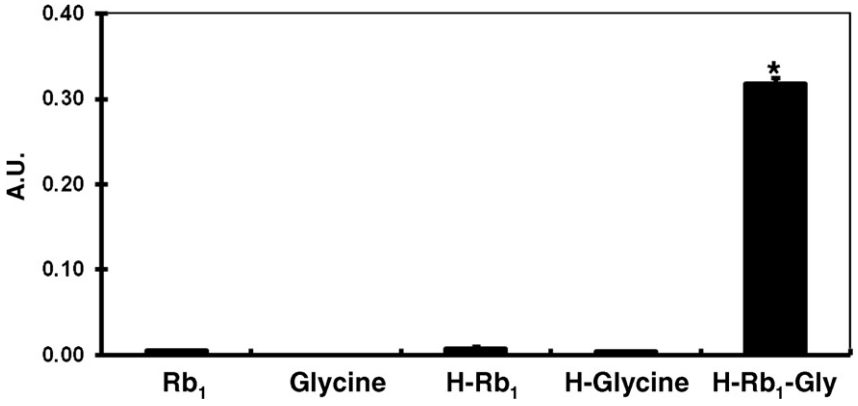

Figure 5. The graph compares the browning compound levels in Rb_1, glycine, heat processed Rb_1, heat processed glycine, and heat processed Rb_1-glycine mixture at the concentration of 0.05%.

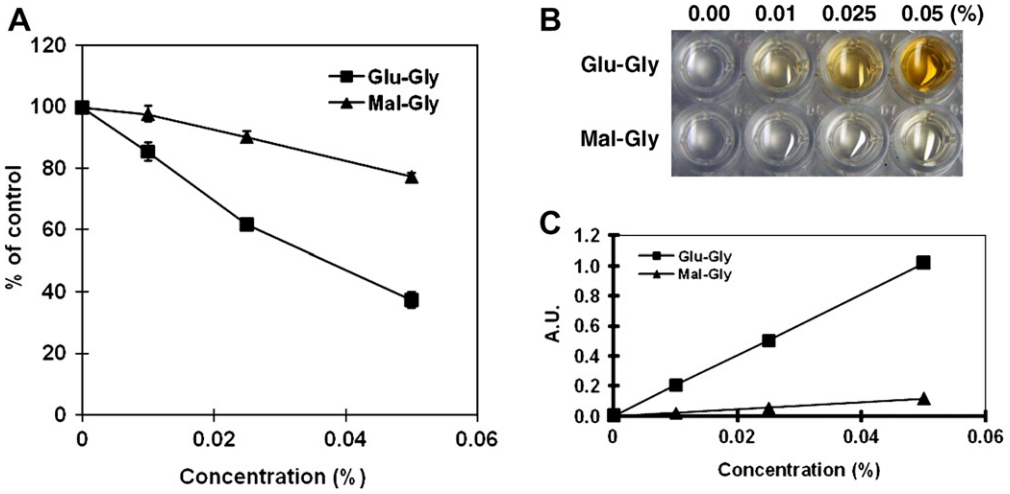

Figure 6. The graphs compare the .OH scavenging activities and browning compound levels of MRPs generated from glucose–glycine and maltose–glycine mixtures. $^{*}p < 0.01$ vs. Rb_1.

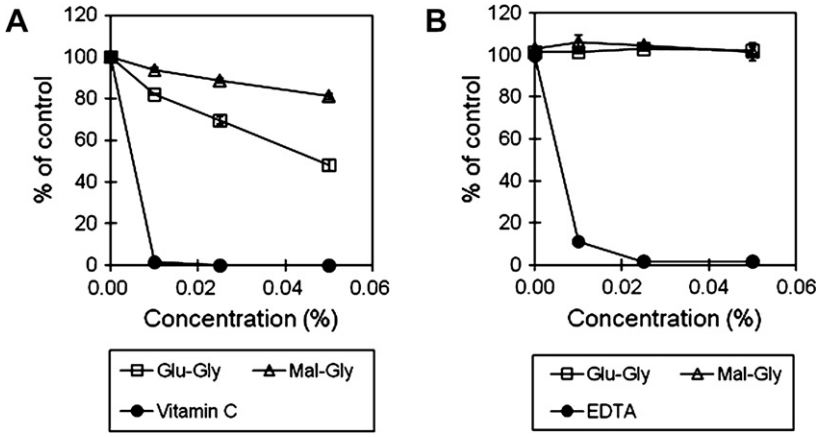

Figure 7. The graphs compare (A) DPPH radical scavenging and (B) ferrous metal ion-chelating activities of MRPs generated from glucose–glycine and maltose–glycine mixtures.

mixture from our previous report were thought to be resulted from the difference in sugar types between Rb_1 and Rb_2.[22] Rb_1 generates glucose, but Rb_2 generates arabinose during heat processing. Although we could not expend our conclusion to the other ginsenosides and amino acids in present study, the identification of indirect antioxidant effect of Rb_1 by generating glucose to make antioxidant MRPs with glycine is novel idea in ginsenoside research.

Subsequently, we examined 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and ferrous metal ion (Fe^{2+})-chelating activities[5] of MRPs from Rb_1-glycine mixture to identify its **.**OH scavenging mechanism. The **.**OH scavenging in present study can be accomplished by direct scavenging of free radical or via prevention of **.**OH formation through the chelation of Fe^{2+}, and the **.**OH inhibiting effect of ginsenoside is known to be mediated by Fe^{2+}-chelating activity.[5] DPPH is a stable free radical, and has been widely used to test the ability of compounds to act as free radical scavengers.[23] In the DPPH radical scavenging activity test of MRPs, MRPs generated from glucose–glycine mixture showed stronger activity than maltose–glycine mixture (Fig. 7(A)). However, both of the MRPs generated from glucose–glycine and maltose–glycine mixtures showed no Fe^{2+}-chelating activity changes (Fig. 7(B)). The correlation between DPPH radical and **.**OH scavenging activities of MRPs was observed (Figs. 6(A) and 7(A)). Therefore, the **.**OH scavenging of MRPs was thought to be mediated by direct free radical scavenging, and it was different from indirect **.**OH inhibition of ginsenoside through Fe^{2+}-chelating.

As shown from the above results, we confirmed that the Rb_1 was gradually changed into 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 by heat processing, and its sugar moieties at carbon-20 were separated (Fig. 3). The **.**OH inhibiting activities of 20(*S*)-Rg_3 and Rg_5 were stronger than that of Rb_1, but 20(*R*)-Rg_3 and Rk_1 showed weak or no **.**OH inhibiting activities. The neutralized **.**OH scavenging activity of these mixtures of strong and weak **.**OH inhibiting ginsenosides of heat processed Rb_1 was not stronger than that of un-treated Rb_1. However, the generated content of 20(*S*)-Rg_3 was higher than that of 20(*R*)-Rg_3 when the Rb_1 was heat processed with glycine, and its browning compound level and **.**OH scavenging activity were significantly increased than that of un-treated Rb_1. Therefore, the increase in **.**OH scavenging activity of Rb_1 by heat processing with glycine was medicated by the generations of 20(*S*)-Rg_3 and MRPs, which have **.**OH scavenging activities (Figs. 4 and 6). The biological and chemical roles of ginsenosides in terms of self-mediated and indirectly mediated actions such as via the Maillard reaction are thought to be valuable in order to understand the complex efficacy changes of ginseng by heat processing.

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17. Rb_1 was isolated and identified from *Panax ginseng* as described in *J. Biochem.* **1975**, *77*, 1057. The molecular formula of Rb_1 was given as $C_{54}H_{92}O_{23}$ from the high-resolution mass spectrum (*m/z* 1109.6115 $[M+H]^+$, calculated for $C_{54}H_{93}O_{23}$ 1109.6115). The same amounts (w/w) of Rb_1 and glycine were steamed together at 120 °C. After drying at 50 °C, un-treated and heat processed Rb_1-glycine mixtures at 120 °C were prepared. The samples were dissolved in distilled water (D.W.)-acetonitrile (1:1, v/v), and the absorbance at 420 nm was measured in a 1 cm glass cuvette using a UV-1200 UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan) to measure the extent of browning. The heat processing and measurement were repeated three times for each sample. The results for each group are expressed as mean ± SE values. Individual differences between groups were evaluated using Student's *t*-test, and those at $p < 0.05$ were considered significant.
18. 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 D.W., v/v) were mixed with 38 μL of 0.2 mM ferrous sulfate and 37 μL of 1 mM diethylenetriaminepentaacetic acid. The mixture was stirred with 30 μL of sample solution and 75 μL of 1 mM hydrogen peroxide. 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 yield of the inhibition of **.**OH by samples. Measurement was repeated three times for each sample. The inhibition of **.**OH was determined by the ratio of peak height of the DMPO-OH spin adduct to the signal of Mn^{2+} and compared to the ratio of the control.

19. The changes in constituents by heat processing were analyzed with a Hitachi (Tokyo, Japan) L-7100 liquid chromatograph fitted with a C-18, reversed-phase column (5 μm, 25 cm × 4.6 mm I.D.; Phenomenex Luna) utilizing the solvent gradient system. 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 evaporative light scattering detector (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.
20. The BuOH fraction of heat processed *Panax ginseng*, as described in *J. Nat. Prod.* **2000**, *63*, 1702, 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 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 were confirmed by comparing the chemical shifts in ^{13}C NMR and ^{1}H NMR with standard samples.
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