

The Chemical and 1,1-Diphenyl-2-Picrylhydrazyl Radical Scavenging Activity Changes of Ginsenosides Rb₁ and Rg₁ by Maillard Reaction

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The chemical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity changes of ginsenoside Rb₁-glycine and ginsenoside Rg₁-glycine mixtures by Maillard reaction were investigated to identify the role of Maillard reaction in the increased antioxidant activity of ginseng by heat-processing. The DPPH radical scavenging activity of Rg₁-glycine mixture was more strongly increased by heat-processing than that of Rb₁-glycine mixture. From the analyses of ginsenosides, Rb₁ was gradually changed into 20(S)-Rg₃, 20(R)-Rg₃, Rk₁ and Rg₅ by heat-processing. Rg₁ was gradually changed into 20(S)-Rh₁, 20(R)-Rh₁, Rk₃ and Rh₄ by heat-processing. However, the generation of these less-polar ginsenosides was not related to the increased DPPH radical scavenging activity of Rb₁-glycine and Rg₁-glycine mixtures because their DPPH radical scavenging activities were already significantly increased when dried at 50°C, which temperature induce no structural changes of ginsenosides. In the comparison of browning compound levels of Rg₁-glycine and Rb₁-glycine mixtures, the extents of Maillard reaction were positively correlated with their increased free radical scavenging activities. Based on the chemical and DPPH radical scavenging activity changes of Rg₁-glycine and Rb₁-glycine mixtures by heat-processing, we clearly identified that the increased free radical scavenging activity of ginsenoside is mediated by the Maillard reaction between sugar moiety of ginsenoside and amino acid.

Keywords: *Panax ginseng*, Ginsenoside Rb₁, Ginsenoside Rg₁, Glycine, 1,1-Diphenyl-2-picrylhydrazyl radical, Maillard reaction

INTRODUCTION

The root of *Panax ginseng* C. A. Meyer (Araliaceae), mainly cultivated in Korea and Northeast China, has been heat-processed to improve its medicinal efficacy in Korea [1,2]. Ginseng root is air-dried to produce white ginseng (WG) or steamed at 98°C to 100°C to yield red ginseng (RG) (Fig. 1) [1,3-5]. RG is more common as a medicinal herb than WG in Asian countries, because steaming induces changes in the chemical constituents and enhances the biological activities [3,5-8]. A novel

heat-processing method of steaming ginseng at the higher temperature than RG was developed to achieve an even stronger efficacy than that of RG, and this ginseng product was termed heat-processed ginseng (HPG) [9-11].

Although the Maillard reaction is known as a major source of compounds related to enhanced antioxidant activity by heat treatment in various crude drugs or foods [12-14], the chemical and free radical scavenging activity changes of ginsenosides brought about by the Maillard

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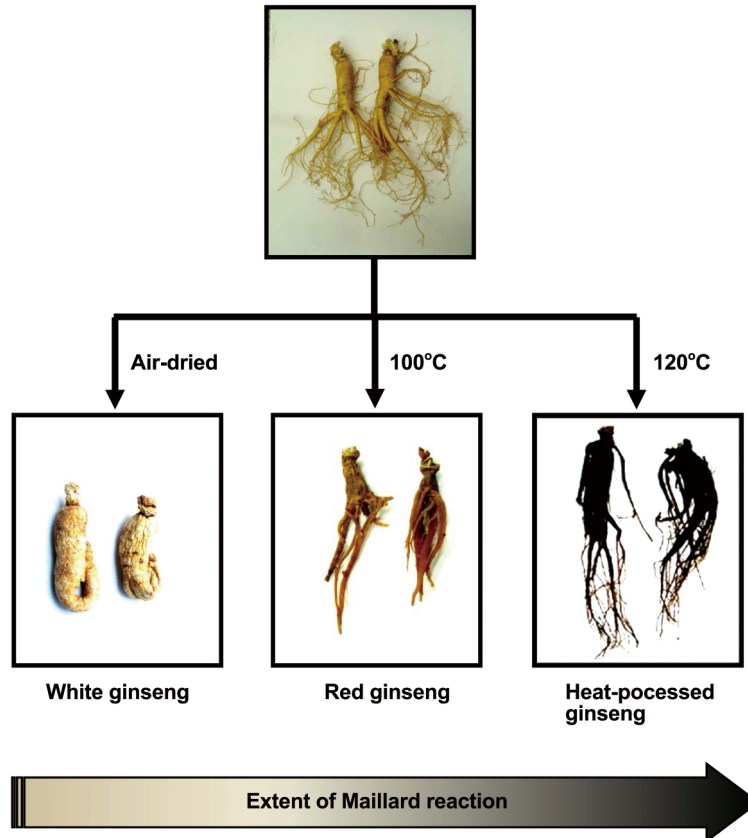


Fig. 1. Preparation of ginseng extracts. Fresh ginseng was purchased from a local ginseng market in Seoul (Korea). White and red ginsengs were purchased from a local ginseng market in Geumsan (Korea). Heat-processed ginseng was prepared as described in materials and methods.

reaction have not been fully elucidated. Ginsenosides have been regarded as the main active components responsible for the pharmacological activities of ginseng [1,2,8,9,15]. Ginsenosides are glycosides of 30-carbon derivatives of the triterpenoid dammarane, as shown in Fig. 2. They have a hydrophobic four-ring steroid-like structure with hydrophilic sugar moieties. More than 30 different types of ginsenoside have been isolated and identified from the root of *Panax* species. Each also has at least two (carbon-3 and -20) or three (carbon-3, -6, and -20) hydroxyl groups, which are free, or bound to monomeric, dimeric, or trimeric sugars [1,8,16].

In our previous studies, we have identified the chemical and antioxidant activity changes of diol-type ginsenosides and amino acid mixture by heat-processing to identify the role of Maillard reaction in the increased free radical scavenging activity of ginseng products as shown in Fig. 1 [17-20]. The critical roles of the Maillard reaction were confirmed and supported by the following lines of observations: Firstly, the generation of Maillard reaction products (MRPs), based on the extent of browning, was positively correlated with the hydroxyl radical (-OH) scavenging activity [20]. Secondly, cer-

tain amino acids such as L-arginine block the structural change of ginsenosides during Maillard reaction which leads to change them to a stronger -OH-scavenging ginsenosides such as 20(*S*)-Rg₃ [21]. Finally, the generated amount of 20(*S*)-Rg₃ from Rb₁ was increased when heat-processed with glycine than without it [21].

The important roles of Maillard reaction in the heat-processing of ginsenosides has been identified with diol-type ginsenosides in our previous studies [18,20,21]. However, little is known about the Maillard reaction of triol-type ginsenosides. The aim of this study was to identify the chemical and antioxidant activity changes of triol-type ginsenoside Rg₁ by Maillard reaction, which has not yet been reported. The change in 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, a widely used method to evaluate antioxidant activity, of Rg₁ by Maillard reaction was compared with Rb₁ as a representative diol-type ginsenoside.

MATERIALS AND METHODS

Chemicals and reagents

DPPH was purchased from Wako Pure Chemical

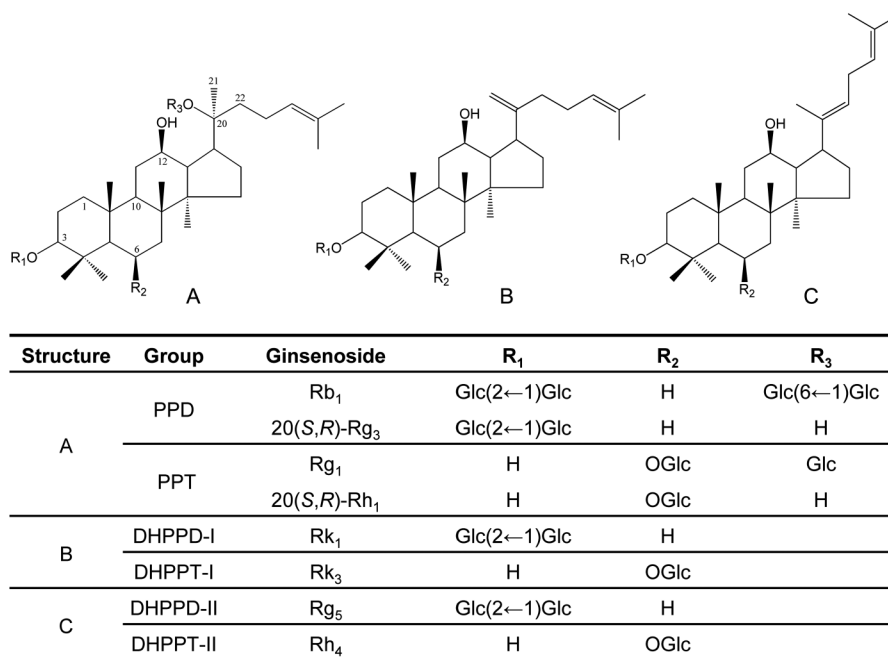


Fig. 2. The chemical structure of ginsenosides. PPD, protopanaxadiol; PPT, protopanaxatriol; DHPPD, dehydroprotopanaxadiol; DHPPT, dehydroprotopanaxatriol; -Glc, D-glucopyranosyl.

Industries Ltd. (Osaka, Japan). Ginsenosides Rb₁ and Rg₁ were isolated and identified from *Panax ginseng* by the reported method [9,22]. The other chemicals and reagents used were of high quality and obtained from commercial sources.

Preparation of ginseng extracts

Four-year-old fresh ginseng (*Panax ginseng*) was purchased from a local ginseng market in Seoul (Korea). The ginseng rootlets were not removed from root body during the preparation of ginseng extracts in this study. WG was produced by drying 100 g of fresh ginseng at 50°C for 3 days. RG was made by steaming WG at 100°C and 0.03 megapascal (MPa) for 3 h, and drying at 50°C for 3 days. HPG was made by steaming WG at 120°C and 0.11 MPa for 3 h, and drying at 50°C for 3 days [18]. The three ginsengs were ground to pass an 80 mesh sieve and extracted under reflux with MeOH three times at 70°C for 2 h, filtered through filter paper (Advantec, Tokyo, Japan), and the solvent was evaporated in vacuo to give a MeOH extract with a yield of 19%, by weight, of the original ginseng powder.

Fractionation of heat-processed ginseng MeOH extract

HPG MeOH extract (30 g) was suspended in water (300 mL) and then the water-soluble polysaccharide fraction was separated by Diaion HP 20 (11 cm×50 cm,

Mitsubishi Chemical, Tokyo, Japan) column chromatography using water (6 L) as an eluting solution, followed by elution with MeOH (2 L) [23]. Each solution was evaporated in vacuo to give the water eluate (27 g) and MeOH eluate (3 g). The MeOH eluate was re-suspended in 500 mL of water and extracted 3 times with 100 mL of ether to yield an ether layer (containing phenolic compounds, polyacetylenes, lipids and sterols) and an aqueous layer. The aqueous layer was then extracted 3 times with 100 mL of *n*-BuOH saturated with water for preparation of the total ginsenosides fraction [24]. Each fraction was dried under reduced pressure and ether (800 mg), *n*-BuOH (2.1 g), and residual fractions (100 mg) were yielded.

Maillard reaction model experiment using ginsenoside and glycine

The same amounts (w/w) of each ginsenosides (Rb₁ and Rg₁) and glycine, a frequently used amino acid in Maillard reaction model experiment, were steamed together at 120°C for 3 h as reported previously [18,21]. After drying at 50°C for 3 days, un-treated and heat processed Rb₁-glycine mixtures at 120°C were prepared. The Rb₁-glycine mixture dried at 50°C for 3 days was also prepared to examine the effect of drying on Maillard reaction.

Measurement of browning compound levels

The extent of browning was measured by the reported

method [25] with minor modification. The samples (5 mg) were dissolved in 1 mL of distilled water (DW)-acetonitrile (1:1, v/v), and the absorbance at 420 nm was measured in a 1 cm glass cuvette using a UV-Vis spectrophotometer (UV-1200; Shimadzu, Kyoto, Japan). Measurement was repeated three times for each sample.

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity test

In microwells, 100 μ L of an aqueous solution of the sample (control: 100 μ L of DW) was added to an ethanolic solution of DPPH (100 μ L, 60 μ M) according to the method of Hatano *et al.* [26]. Four concentrations were prepared for each sample. After mixing gently and leaving to stand for 30 min at room temperature, the absorbance at 540 nm was determined using a microplate reader (Tecan SPECTRAFluor; Tecan UK, Goring-on-Thames, UK), and a vitamin C was used as DPPH scavenging positive control compound.

HPLC analyses of ginsenosides

The changes in constituents by Maillard reaction were analyzed with a Hitachi (Tokyo, Japan) L-7100 liquid chromatograph fitted with a C-18, reversed-phase column (5 μ m, 25 cm \times 4.6 mm internal diameter; Phenomenex

Luna, Torrance, CA, USA) 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, Alfortville, 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 [10,27].

Data analysis

Results were expressed as mean \pm SE. The statistical significance was determined by analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Comparison in the browning compound levels and DPPH radical scavenging activities of ginseng extracts and sub-fractions of HPG

Fig. 3 shows the comparisons in the extent of Maillard reaction (browning compound levels) and DPPH radical scavenging activities of ginseng extracts and sub-fractions of HPG. The browning compound lev-

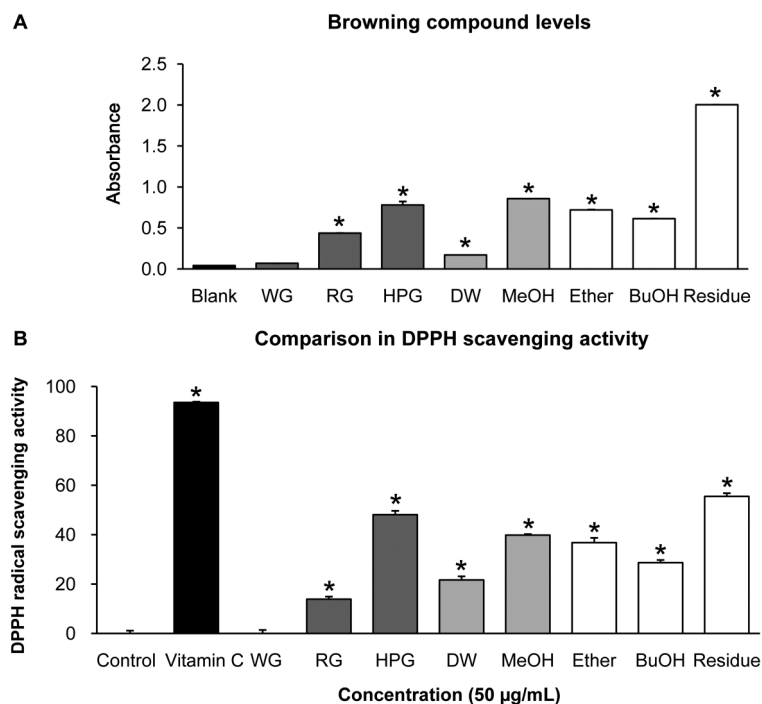


Fig. 3. The graphs compare (A) the browning compound levels and (B) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of ginseng extracts and sub-fractions of heat-processed ginseng (HPG). WG, white ginseng; RG, red ginseng; DW, distilled water. *p*<0.05 compared to the blank or control value.

els of ginsengs were increased in the heat-processing temperature-dependent manner (Fig. 3A). The browning compound level of HPG was higher than those of WG and RG. Among the sub-fractions of HPG, the browning compound level of MeOH eluate was 5 times higher than that of water eluate (Fig. 3A). MeOH eluate was further separated into three sub-fractions. Of these, the residual fraction showed the highest browning compound level, followed by the ether fraction, and then the *n*-BuOH fraction containing ginsenosides (Fig. 3A).

As shown in Fig. 3B, the three ginseng extracts exhibited distinctive DPPH radical scavenging activities. HPG showed the strongest activity, followed by RG and then WG. The DPPH radical scavenging activity of ginseng was increased by heat-processing in a temperature-dependent manner. After separation of the HPG extract,

significant differences among the scavenging activities of the various fractions were observed. The scavenging activity of MeOH eluate was stronger than that of water eluate. Among the sub-fractions of MeOH eluate, the residual fraction showed the strongest DPPH radical scavenging activity, followed by the ether fraction, and then the *n*-BuOH fraction (Fig. 3B).

Comparison in the DPPH radical scavenging activities of Rb₁-glycine and Rg₁-glycine mixtures

Fig. 4 shows the comparisons in the DPPH radical scavenging activities of Rb₁-glycine and Rg₁-glycine mixtures. Rb₁-glycine and Rg₁-glycine mixtures only slightly decreased the DPPH radical generation with increasing concentrations. The Rb₁-glycine mixtures dried at 50°C and heat-processed at 120°C increased

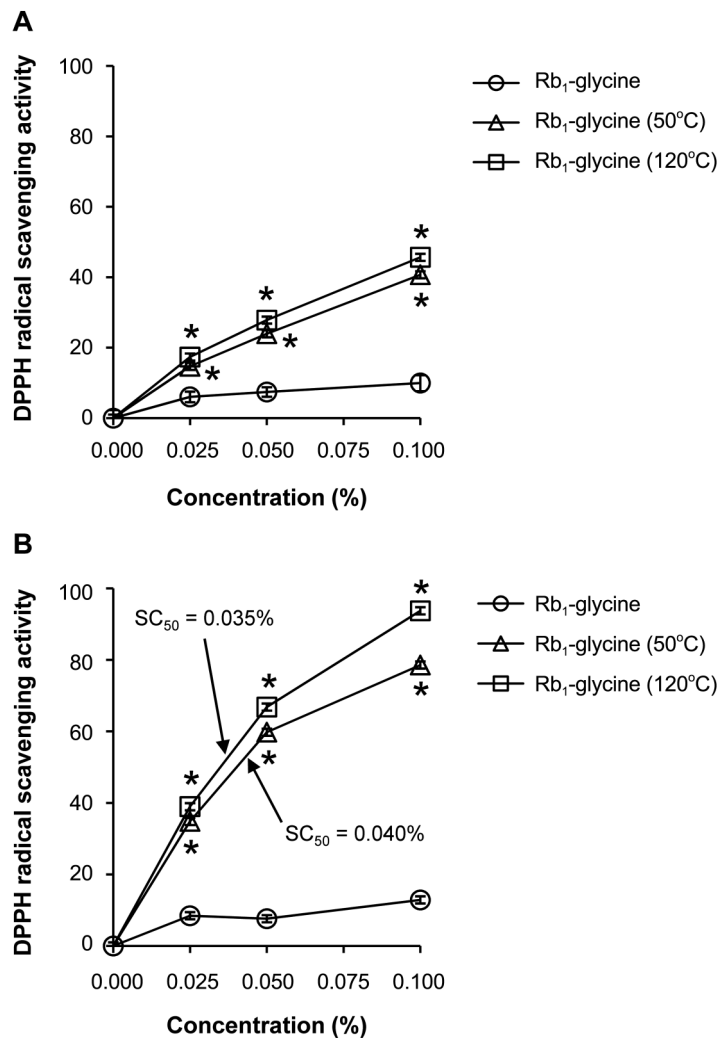


Fig. 4. The comparisons in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of (A) Rb₁-glycine mixtures and (B) Rg₁-glycine mixtures. *p < 0.05 compared to the not-treated value.

the DPPH radical scavenging activity to 41% and 46%, respectively, at the concentration of 0.1% (Fig. 4A). In the case of Rg₁-glycine mixtures, Rg₁-glycine mixtures dried at 50°C and heat-processed at 120°C increased the DPPH radical scavenging activity to 79% and 94%, respectively, at the concentration of 0.1% (Fig. 4B). The DPPH radical scavenging activity of Rg₁-glycine mixtures was more strongly increased by heat-processing than those of Rb₁-glycine mixtures (Fig. 4).

HPLC chromatograms of Rb₁-glycine and Rg₁-glycine mixtures

As shown in the HPLC chromatograms of Rb₁-glycine

mixture, glycine and Rb₁ were detected at 3.0 and 18.4 min, respectively, when not steamed (Fig. 5A) and its content was slightly decreased when dried at 50°C (Fig. 5B). The peak of Rb₁ was completely disappeared and the contents of 20(*S*)-Rg₃, 20(*R*)-Rg₃, Rk₁ and Rg₅ which detected at 29.8, 31.0, 38.5 and 39.1 min, respectively, were increased by heat-processing at 120°C (Fig. 5C). In the case of Rg₁-glycine mixture, Rg₁ was detected at 14.0 min when not steamed (Fig. 5D) and its content was slightly decreased when dried at 50°C (Fig. 5E). The peak of Rg₁ was completely disappeared and the contents of 20(*S,R*)-Rh₁, Rk₃ and Rh₄ which detected at 20.4, 21.0, 30.2 and 31.3 min, respectively, were in-

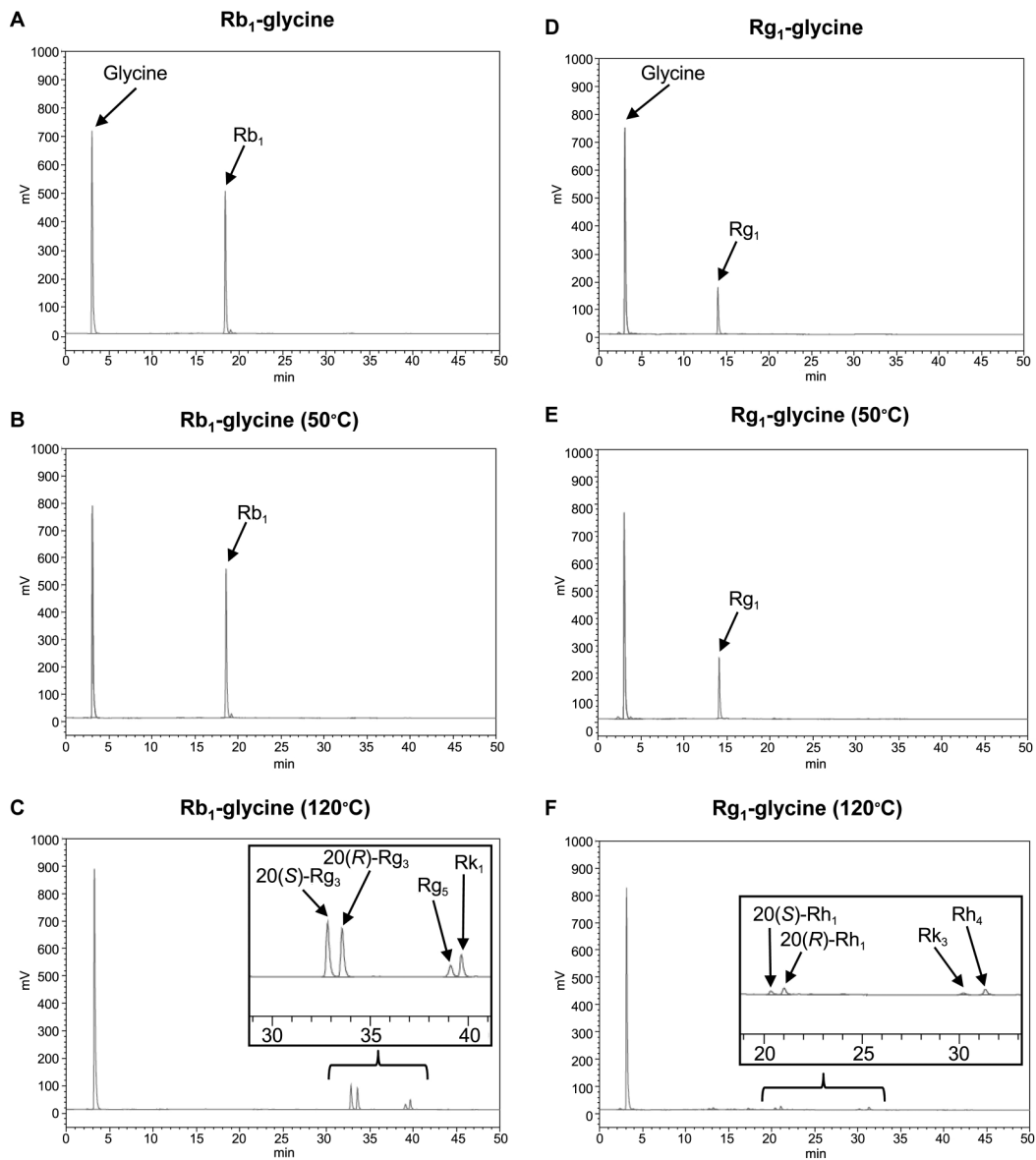


Fig. 5. HPLC chromatograms of Rb₁-glycine mixtures (A-C) and Rg₁-glycine mixtures (D-F).

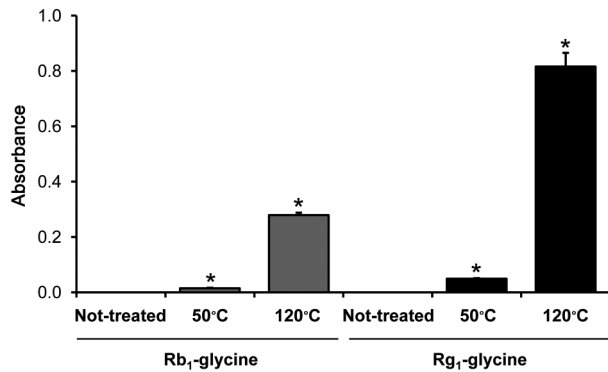


Fig. 6. The graphs compare the browning compound levels of Rb₁-glycine and Rg₁-glycine mixtures. **p*<0.05 compared to the not-treated value.

creased by heat-processing at 120°C (Fig. 5F).

Comparison in the browning compound levels of Rb₁-glycine and Rg₁-glycine mixtures

Fig. 6 shows the changes in browning compound levels of Rb₁-glycine and Rg₁-glycine mixtures by Maillard reaction. Rb₁-glycine and Rg₁-glycine mixtures showed no changes in browning compound levels when not heat-processed. After drying at 50°C, the browning compound level of Rg₁-glycine mixture was increased 3.3 times higher than that of Rb₁-glycine mixture. When heat-processed at 120°C, the browning compound levels of Rb₁-glycine and Rg₁-glycine mixtures were increased to 19 and 16.6 times higher values, respectively, than those of dried at 50°C. The browning compound level of Rg₁-glycine mixture was 2.9 times higher than that of Rb₁-glycine mixture when heat-processed at 120°C (Fig. 6).

DISCUSSION

The Maillard reaction of sugars with amino acids is a nonenzymatic browning reaction that takes place during the processing, cooking, and storage of foods. It is well known that MRPs produced in both heat-treated food systems and in sugar-amino acid model systems have antioxidant activity [12-14]. The Maillard reaction occurs in the processing of RG [28]. MRPs in ginseng were reported to increase by heat-processing; these compounds are arginyl-fructosyl-glucose, arginyl-fructose, maltol, maltol-3-*O*-β-D-glucoside, etc. [28,29]. To date, it is not clear what Maillard reaction compounds contribute to the antioxidant activity of MRPs, and how this activity develops over time [14]. Therefore, we have investigated the serial Maillard reaction model experiments using ginsenosides and amino acids to investigate the factors which would lead to an increase in the free

scavenging activity of ginseng.

We first compared the browning compound levels and DPPH radical scavenging activities of ginseng extracts and sub-fractions of HPG. The browning compound levels and DPPH radical scavenging activities of ginseng extracts were increased in the heat-processing temperature-dependent manner (Fig. 3). *Panax ginseng* consists of various components which need to be separated into fractions for the identification of core active components. In this study, a water-soluble fraction (containing mainly polysaccharides) of HPG was separated by Diaion HP 20 column chromatography using water as an eluting solution, followed by elution with MeOH soluble residues (containing saponins, phenolic compounds, etc). MeOH soluble fractions showed a stronger DPPH radical scavenging activity and higher browning compound levels than water eluate (Fig. 3), and MeOH eluate was further separated into total ginsenosides, phenolic compounds, and residual fractions by the reported extraction methods [23,24]. Among the sub-fractions of MeOH eluate, the residual fraction showed the highest browning compound level and the strongest DPPH radical scavenging activity. Therefore, the free radical scavenging activity of ginseng was suggested to be closely correlated with the extent of Maillard reaction.

As shown in Fig. 4, the DPPH radical scavenging activity of Rg₁-glycine mixture was more strongly increased by heat-processing than those of Rb₁-glycine mixture. In the analyses of ginsenosides by HPLC, Rb₁ was gradually changed into 20(*S*)-Rg₃, 20(*R*)-Rg₃, Rk₁ and Rg₅ by heat-processing. Rg₁ was gradually changed into 20(*S*)-Rh₁, 20(*R*)-Rh₁, Rk₃ and Rh₄ by heat-processing. However, the generation of these less-polar ginsenosides was not related to the increased DPPH radical scavenging activity of Rb₁-glycine and Rg₁-glycine mixtures because their DPPH radical scavenging activities were already increased when dried at 50°C, but no structural change of ginsenosides occurs at this temperature (Figs. 4 and 5B, E). From the comparisons of browning compound levels and DPPH radical scavenging activities of Rg₁-glycine and Rb₁-glycine mixtures (Figs. 4 and 6), the free radical scavenging activity of ginsenosides were positively correlated with the extents of Maillard reaction. During the Maillard reaction of Rb₁ and Rg₁, glucoses are separated, respectively, from Rb₁ and Rg₁ (Fig. 2). We have previously identified that the generation of browning compound from glucose-glycine mixture is significantly higher than that of maltose-glycine mixture [20]. Therefore, the stronger DPPH radical scavenging activity and MRPs of Rg₁-glycine mixture

than Rb₁-glycine mixture was resulted to be mediated by the difference in types of sugar moieties in the structure of ginsenosides.

Based on the chemical and DPPH radical scavenging activity changes of Rg₁-glycine and Rb₁-glycine mixtures by heat-processing, we clearly identified that the increased free radical scavenging activity of ginsenoside by Maillard reaction is dependent on the type of sugar moieties in the structure of ginsenosides. The chemical roles of ginsenosides in terms of self-mediated and indirectly mediated actions such as via the Maillard reaction are thought to be a valuable in order to understand the complex efficacy changes of ginseng by heat-processing.

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