The Effects of Glycine and L-Arginine on Heat Stability of Ginsenoside Rb₁

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To identify the effects of amino acids on the heat stability of ginsenoside Rb₁ (Rb₁), Rb₁ was heat-processed at 120 °C with or without glycine or L-arginine. Rb₁ was changed into 20(S)-Rg₃, 20(R)-Rg₃, Rk₁, and Rg₅ by heat-processing through glycosyl elimination and epimerization of carbon-20 by SN1 reaction. Similarly, Rb₆ was changed into 20(S)-Rg₃, 20(R)-Rg₃, Rk₁, and Rg₅ when it was heat-processed with the same amount of glycine, but the generated amount of 20(S)-Rg₃ was higher than when Rb₁ was heat-processed without amino acids, and a significant increase in Maillard reaction products (MRPs) was noted. On the other hand, there were no structural changes in Rb₁ and the generation of MRPs when Rb₁ was heat-processed with the same amount of L-arginine. The improved heat stability of Rb₁ brought about by the addition of L-arginine was thought to be closely related to its characteristics of interfering with nonenzymatic glycation and forming hydrogen bonds with Rb₁.

Key words ginsenoside Rb₁; glycine; L-arginine; heat-processing; heat stability

The root of ginseng, Panax ginseng C. A. Meyer (Araliaceae), has been heat-processed to improve its medicinal efficacies in Korea based on the long history of ethnopharmacological evidence. Although the increasing body of evidence supports that Maillard reaction products (MRPs) are implicated in the increased activity by heat treatment in various crude drugs or foods, the effect of Maillard reaction on the active components of ginseng and biological activities have not yet been fully elucidated. Ginsenosides have been regarded as the main active components responsible for the pharmacological activities of ginseng, and are well-known to be deglycosylated by heat-processing. The sugar moieties of ginsenosides can be a source of MRPs with amino acids contained in ginseng during heat-processing, and research on the Maillard reaction of ginsenosides is thought to be beneficial to understand the complex structural changes of ginsenosides brought about during the heat-processing of ginseng.

As one of the major ginsenosides contained in Panax ginseng, ginsenoside Rb₁ (Rb₁) is a diol-type triterpene glycoside, and it is known to exhibit anti-inflammatory, antioxidative, analgesic, and antiplatelet effects, promote the synthesis of protein, nucleic acid, and cholesterol, and inhibit neural lipid breakdown. At the same time, the heat-processing-induced deglycosylation of two glucose molecules at carbon-20 of Rb₁ has been well documented. Therefore, Rb₁ was used as a target ginsenoside to study a Maillard reaction model experiment in this study.

To identify the effects of amino acids on the heat stability or structural changes of Rb₁, Rb₁ was heat-processed with or without the same amount of glycine or L-arginine, because glycine is a frequently used amino acid in Maillard reaction model experiments and L-arginine is the most abundant amino acid in Maillard reaction (70 eV). A medium polarity column, DB-17 capillary column (30 m x 0.25 mm, 0.25 µm, J&W Scientific, U.S.A.) was employed, and helium was used as the carrier gas at a flow rate of 1 ml/min. The injection port temperature was 240 °C, and the oven was programmed to remain at 50 °C for 3 min and to raise the temperature from 50 to 170 °C at a rate of 5 °C per min and from 170 to 270 °C at a rate of 8 °C per min, and finally hold at 270 °C for 20 min.

HPLC and GC-MS Analyses Changes in ginsenosides brought about by heat-processing were analyzed with a Hitachi (Tokyo, Japan) L-7100 liquid chromatograph fitted with a C-18, reverse-phase column (5 µm, 25 cm x 4.6 mm 1.5 µm; 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. The analysis was repeated three times for the verification of repeatability.

On the other hand, the components of samples were analyzed using GC-MS by the reported method with minor modifications. The samples were trimethylsilylated (TMS) before GC-MS analysis. The TMS derivatives of the samples were separated and analyzed on a Hewlett-Packard HP6890 (Agilent Technologies, Palo Alto, CA, U.S.A.) GC coupled to a JEOL JMS-600W (Jeol Ltd., Tokyo, Japan). Mass spectra were measured in electron impact ionization mode (70 eV). A medium polarity column, DB-17 capillary column (30 m x 0.25 mm, 0.25 µm, J&W Scientific, U.S.A.) was employed, and helium was used as the carrier gas at a flow rate of 1 ml/min. The injection port temperature was 240 °C, and the oven was programmed to remain at 50 °C for 3 min and to raise the temperature from 50 to 170 °C at a rate of 5 °C per min and from 170 to 270 °C at a rate of 8 °C per min, and finally hold at 270 °C for 20 min.

MATERIALS AND METHODS

Steaming Model Experiment Using Rb₁, Rb₁-Glycine, and Rb₁-Arginine Mixtures Rb₁ (Fig. 1) was isolated and identified from Panax ginseng by the reported method. The molecular formula of Rb₁ was given as C₅₄H₉₂O₂₃ from the high-resolution mass spectrum (m/z 1109.6115 [M+H]+, calculated for C₅₄H₉₂O₂₃ 1109.6115). One milligram of Rb₁ was autoclaved with or without the same amounts (w/w) of glycine or L-arginine (free form, Wako Pure Chemical Industries Ltd., Osaka, Japan) in microcentrifuge tube (2.0 ml) containing 200 µl of distilled water. After drying at 50 °C, heat-processed Rb₁, Rb₁-glycine, and Rb₁-arginine mixtures were prepared.
Measurement of the MRP Level  The extent of browning was measured by the reported method\textsuperscript{15} with minor modifications. Rb\textsubscript{1}, Rb\textsubscript{1}-glycine, and Rb\textsubscript{1}-arginine mixtures and their steamed products at 120 °C were dissolved in 70% MeOH (5 mg/ml), and the absorbance values at 420 nm were measured in a 1 cm glass cuvette using a UV-1200 UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan). Measurement was repeated three times for each sample.

Statistical Analysis  The results for each group are expressed as mean±S.E. values. Individual differences between groups were evaluated using Student’s \textit{t}-test, and those at \( p < 0.05 \) were considered significant.

RESULTS AND DISCUSSION

Ginsenosides are known as the main pharmacologically active constituents of ginseng, and consist of four hydrophobic ring steroid-like structures with hydrophilic sugar moieties. Dimeric sugars are bound to hydroxyl groups (–OH) on carbon-3 and -20 of diol-type ginsenosides as shown in Fig. 1. They also exist as stereoisomers: 20(\textit{S})-ginsenosides and 20(\textit{R})-ginsenosides are epimers of each other depending on the geometrical position of –OH on carbon-20. This epimerization is known to occur by the selective attack of OH\textsuperscript{−} after the elimination of the glycosyl residue at carbon-20 during the steaming process.\textsuperscript{2,16} In addition, more less-polar ginsenosides such as Rk\textsubscript{1} and Rg\textsubscript{5} are known to be easily produced by the elimination of H\textsubscript{2}O at carbon-20 of Rg\textsubscript{3} under high pressure and temperature conditions, like in autoclaving.\textsuperscript{3} As shown in Figs. 2A and B, Rb\textsubscript{1} (1000 \( \mu \)g) was changed into 20(S)-Rg\textsubscript{3} (146 \( \mu \)g), 20(R)-Rg\textsubscript{3} (201 \( \mu \)g), Rk\textsubscript{1} (102 \( \mu \)g), and Rg\textsubscript{5} (110 \( \mu \)g) by heat-processing (Table 1), and the sugar moieties at carbon-20 of Rb\textsubscript{1} were deglycosylated, as mentioned above. The separated sugar moiety was determined as glucose from GC-MS analysis (data not shown). Then, we added the same amount of glycine to Rb\textsubscript{1} to identify the effect of the Maillard reaction during heat-processing. Rb\textsubscript{1} (1000 \( \mu \)g) was changed into 20(S)-Rg\textsubscript{3} (196 \( \mu \)g), 20(R)-Rg\textsubscript{3} (167 \( \mu \)g), Rk\textsubscript{1} (102 \( \mu \)g), and Rg\textsubscript{5} (108 \( \mu \)g) when heat-processed with glycine (Figs. 2C, D), and the brown color level of heat-processed Rb\textsubscript{1}-glycine mixture was significantly higher than Rb\textsubscript{1} or heat-processed Rb\textsubscript{1} (Fig. 3). Maillard reaction is dependent on several factors such as pH, time, temperature, the concentration of reactants, and reactant type. The development of color is known as an important and obvious feature of the Maillard reaction, and brown-colored nitrogenous polymers, called melanoidins, are known to be formed by this reaction.\textsuperscript{15,17} When changes in the contents of ginsenosides between heat-processed Rb\textsubscript{1} and heat-processed Rb\textsubscript{1}-glycine mixture were compared, the generated amounts of 20(S)-Rg\textsubscript{3} and 20(R)-Rg\textsubscript{3} were thought to be inverse in these samples (Figs. 2B, D, Table 1). On the other hand, the pH values of Rb\textsubscript{1} and Rb\textsubscript{1}-glycine mixture were about 4.51 and 5.28, respectively. The pH value of the same amount of ginseng extract was about 5.35. The exact mechanism to explain the effect of glycine on the epimerization of ginsenoside is not certain at present, but the involvement of the Maillard reaction is certain. Therefore, the addition of glycine to Rb\textsubscript{1} in heat-processing was thought to increase the generation of 20(S)-ginsenoside by the Maillard reaction.

At the same time, when Rb\textsubscript{1} was steamed with the same amount of L-arginine, about 0.5% of Rb\textsubscript{1} was lost during heat-processing, but the heat stability of Rb\textsubscript{1} was significantly improved (Figs. 2E, F, Table 1) compared to when Rb\textsubscript{1} was heat-processed with or without the same amount of glycine. In addition, there was no increase in the brown color.
by heat-processing of the Rb₁-arginine mixture (Fig. 3), and the pH value of Rb₁-arginine mixture was about 10.37. The high temperature and pH is known to promote Maillard reaction, and the L-arginine is most abundant amino acid in *Panax ginseng* to make MRPs such as arginyl-fructose and arginyl-fructosyl-glucose.\(^{13,18,19}\) However, the Maillard reaction was not occurred when Rb₁ was steamed with L-arginine, and we paid attention to the structural characteristics of L-arginine. The substitution of L-arginine in protein is known to lead to significant heat stability enhancement in the pres-

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<th>Rb₁</th>
<th>20(S)-Rg₃</th>
<th>20(R)-Rg₃</th>
<th>Rk₁</th>
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<td>Untreated Rb₁</td>
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<td>Heat-processed Rb₁</td>
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<td>201</td>
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<td>Heat-processed Rb₁–glycine mixture</td>
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<td>196</td>
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<td>Rb₁–arginine mixture</td>
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<td>Heat-processed Rb₁–arginine mixture</td>
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Data are expressed as µg of sample.
ence of sugar substrates, most probably by interfering with nonenzymatic glycation. In addition, the guanidyl groups of L-arginine generally forms long-range hydrogen bonds or electrostatic interactions with negatively charged groups, and this increased hydrogen bonding is one of the factors enhancing protein thermostability. Therefore, the improved heat stability of Rb1 was thought to be closely related to its characteristics of interfering with nonenzymatic glycation and forming hydrogen bonds with Rb1. However, we still have unanswered questions and need to conduct more precisely controlled examinations using other amino acids or using similar pH conditions with ginseng to prove the detailed mechanism behind these reactions.

In summary, Rb1 was changed into 20(S)-Rg3, 20(R)-Rg3, Rk1, and Rg5 by heat-processing through glycosyl elimination and epimerization of carbon-20 by SN1 reaction (Fig. 4). Similarly, Rb1 was changed into 20(S)-Rg3, 20(R)-Rg3, Rk1, and Rg5 when it was heat-processed with the same amount of glycine, but the generated amount of 20(S)-Rg3 was higher than when Rb1 was heat-processed without amino acids, and a significant increase of MRPs was noted. On the other hand, there were no structural changes in Rb1 and the generation of MRPs when Rb1 was heat-processed with the same amount of L-arginine. The improved heat stability of Rb1 brought about by the addition of L-arginine was thought to be closely related to its characteristics of interfering with nonenzymatic glycation and forming hydrogen bonds with Rb1. Therefore, the generated ratios of 20(S)- and 20(R)-ginsenosides and heat stability of ginsenosides through heat-processing were thought to be partially related to the roles of certain amino acids such as glycine and L-arginine contained in ginseng.

REFERENCES


Fig. 4. Structural Changes in Rb1 by Heat-Processing with Glycine or L-Arginine.