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Thermal Conversion Pathways of Ginsenosides in Red Ginseng Processing

Sang Myung Lee*

Korea Ginseng Corp., Central Research Institute, 305-805 Daejeon, Republic of Korea

Abstract – According to the results of my study on the chromatographic analysis of fresh ginseng (*Panax ginseng* C. A. Meyer) roots, most of the contents of protopanxadiol ginsenosides Rb_1, Rc, Rb_2, and Rd are derived from the corresponding malonyl ginsenosides in fresh ginseng by a heat process. Also, I confirmed that acetyl ginsenosides are naturally occurring constituents in fresh ginseng, not decarboxylates from malonyl ginsenosides. Seven neutral ginsenosides Rg_1, Re, Rf, Rc, Rb_1, Rb_2, and Rd were transformed to specific conversions in red ginseng preparation conditions. The conversion paths progress by three rules concluded from my study. These conversion rules are **I**: the ether bond is stable at positions 3 and 6 in the dammarane skeleton, **II**: the ether bond between sugars is stable in glycosides, and **III**: the ether bond to glycosides is unstable at position 20 in the dammarane skeleton.
Keywords – *Panax ginseng*, Malonyl-ginsenoside, Neutral-ginsenoside, Converted ginsenoside, Conversion pathway

Introduction

Ginseng, the root of *Panax ginseng* C. A. Meyer, has been used to improve immunity, alleviate fatigue, recover energy, and increase resistance to environmental stress.[1-3] A variety of commercial ginseng products are available, including fresh ginseng root, white ginseng, and red ginseng. White ginseng is prepared from fresh ginseng root by dehydration, but red ginseng is prepared by steaming fresh ginseng root at 95 - 100 °C for 2-3 h.[4] Dammaran ginsenosides are characteristic components of ginseng and are considered to be the primary pharmacologically effective components in ginseng. Thus far, about 50 kinds of ginsenosides have been identified from the ginseng root, which are defined as protopanaxadiol, and protopanaxtriol according to dammarane skeleton.[5-8] New ginsenosides are continuing to be discovered. However, most of the newly found ginsenosides are converted ginsenosides produced by heat treatment and, acid treatment, or transformation by microbes from naturally occurring ginsenosedes. In particular, red ginseng is prepared by heat treatment, so it has relatively high concentration of the conversions transformed from naturally occurring ginsenosides. The conversion ginsenosides Rg_2, Rg_6, F_4, 20(E)-F_4, Rh_1, Rh_4, Rk_3, Rg_3, Rg_5, Rz_1, Rk_1, Rg_9, and Rg_{10} were found in red ginseng, and these are converted from major ginsenosides Rb_1, Rb_2, Rc, Rd, Rg_1, and Re. Unfortunately, the major ginsenosides as a neutral form, commonly known as naturally occurring substances, are artifacts in a large part of the contents. It is not clear how to distinguish between naturally occurring ginsenosides and artificial converted ginsenosides because these converted ginsenosides are often found in trace amounts in white ginseng, and the contents of naturally occurring ginsenosides are irregular due to sample preparation methods, even for the same sample.[9] According to previous papers,[10-11] the true ginsenoside content of ginseng was being underestimated by ignoring malonyl ginsenosides, which can constitute a substantial proportion of the total ginsenoside content. Malonyl ginsenosides, which are thermally unstable, can degrade into corresponding neutral ginsenosides. A portion of the content of ginsenoside Rb_1, the most important marker substance of ginseng together with Rg_1, originated from the demalonylation of malolnyl ginsenosides. In addition, some portions of the content of neutral ginsenosides are degraded into other conversion ginsenosides in thermal conditions. Therefore, there are needs to define the naturally occurring ginsenosides from strictly selected fresh ginseng and to determine the conversion pattern of ginsenosides for predicting the kind of ginsenosides in ginseng preparations.

I studied the contents of neutral ginsenosides in fresh ginseng and the relevance to malonyl ginsenosides. In addition, I determined the rules for overall ginsenoside

*Author for correspondence
Sang Myung Lee, Korea Ginseng Corp., Central Research Institute, 305-805 Daejeon, Republic of Korea
Tel: +82-42-870-3033; E-mail: pumoripu@gmail.com

conversion paths by the results of conversion reaction of authentic ginsenosides, and present the conversion progress of protopanaxatriol and protopanaxadiol ginsenosides.

Experimental

General experimental procedures – Six-year cultivated fresh *P. ginseng* roots were kindly provided from the Korea Ginseng Corporation (KGC, Daijon Korea). Ginsenosides Rg_1, Re, Rf, Rb_1, Rc, Rb_2, and Rd were used as initial substances to study the degradation patterns, and conversion ginsenosides Rg_2, Rg_6, Rg_9, Rg_{10}, Rh_1, Rk_3, F_4, Rh_4, Rk_1, and Rg_5 were isolated and identified by ^1H, ^{13}C NMR, UPLC/Q-TOF-MS spectroscopy in our laboratory. HPLC-MS-grade acetonitrile was purchased from Merck Co. (Merck, Darmstadt, Germany). Deionized water was purified by a Mili-Q system (Millipore, Bedford, MA, USA). Mass-spectra-grade formic acid was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). BioUltra-grade citric acid was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Instruments – Contents analysis was performed on a Waters Alliance HPLC system with a 2695 Separation Module, a 996 PDA Detector (Waters, MA, Milford, USA), and an Alltech 3300 ELDS (Alltech, Nicholasville, KY, USA). Empower Pro Chromatography Data Software (Waters, MA, Milford, USA) was used for the data acquisition and processing as well as for regulating the HPLC system. Chromatographic separations were performed on an ODS column (4.6 mm ID × 250 mm, 5 μm, Discovery C18, SUPELCO, Bellefonte, PA, USA). In order to confirm the peak identity of acetyl ginsenoside, a Waters ACQUITY UPLC system coupled with a Q-TOFMS Xevo Detector (Waters, Milford, MA, USA) was used. The column used for this specific purpose was an Acuquity BEH C_{18} (2.1 mm ID × 100 mm, 1.7 μm, Waters, Milford, MA, USA).

Analysis of malonyl ginsenosides by HPLC/ELSD – Analyses were performed on a Waters HPLC system (Alliance HPLC system with 2695 Separation Module) equipped with an evaporative light-scattering detector (Alltech 3300 ELDS) at a nebulizer temperature of 70 °C with air as the nebulizing gas with a flow rate of 2.0 L/min. The column (Discovery C18, SUPELCO) temperature was kept at a constant temperature of 40 °C, and the mobile phase flow rate was 1.6 mL/min. The mobile phases consisted of water (8 mM ammonium acetate at pH 7.0 with ammonium hydroxide with reference to Fuzzati) (A) and acetonitrile (B) using a gradient elution of 20 - 32% B at 0 - 40 min, 32 - 50% B at 40 - 55 min, and 50 - 65% B at 55 - 70 min. The re-equilibration time for the mobile phase was 10 min. The content of mRb_1 was approximated from a regression equation by the content of $Rb_{1,6h} - Rb_{1,0h}$ (x), the peak area of $mRb_{1,0h} - mRb_{1,6h}$ (y), and 0 as a y intercept. The contents of mRc, mRb_2, and mRd were approximated by the same method of mRb_1 but calculated from the total value of three peak areas and total content of corresponding neutral ginsenosides.

Analysis of dehydrated ginsenosides by HPLC/PDA – Analyses were performed on an Alliance HPLC system with a 2695 Separation Module equipped with a 996 PDA Detector (Waters, MA, Milford, USA). The column (Discovery C18, SUPELCO) temperature was kept at a constant temperature of 40 °C, and the mobile phase flow rate was 1.6 mL/min. The detection wavelength was 203 nm. The mobile phases consisted of water (A) and acetonitrile (B) using the same gradient elution of as in the malonyl ginsenoside analysis method.

Analysis of acetylginsenosides by UPLC/Q-TOF-MS – All ginsenoside peaks were identified by UPLC/Q-TOF Mass spectroscopy. Chromatographic separation was performed on an ACQUITY UPLC BEH C_{18} column (50 mm × 2.1 mm i.d., 1.7 μm, Waters, USA) at 40 °C. The two mobile phases were phase A (water-formic acid, 100 : 0.01, v/v) and phase B (acetonitrile-formic acid, 100 : 0.01, v/v), and the proportion of B was kept at 15% for 2 min and then gradually increased to 90% of B for 40 minutes. The flow rate was kept at 0.4 mL/min, and 3 μL of the sample solution was injected in each run. The mass spectrometry was performed on a quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Waters Q-TOF Xevo). The nebulizer gas was set to 50 L/h at a temperature of 400 °C under negative ion mode. The cone gas temperature was set to 120 °C. The capillary voltages were set to 2.5 kV, and the cone voltages were set to 25 V.

Sample preparation to study of degradation of malonyl and acetyl ginsenosides – In order to observe each ginsenoside change by the heating time of fresh ginseng, finely crushed fresh ginseng were divided into 2-g samples in seven gas-tight containers, followed by 0, 1, 2, 3, 4, 5, and 6 h of sequential heating at 100 °C. Each sample was rapidly cooled to −75 °C after the end of heating. The samples (2 g) were extracted by ultrasonication in 20 mL of methanol for 0.5 h, filtered by a 0.2-μm membrane disk filter, and analyzed by HPLC/ELSD and UPLC-Q-TOF promptly.

Sample preparation to study of dehydration pattern of neutral ginsenosides – To convert ginsenosides Rg_1,

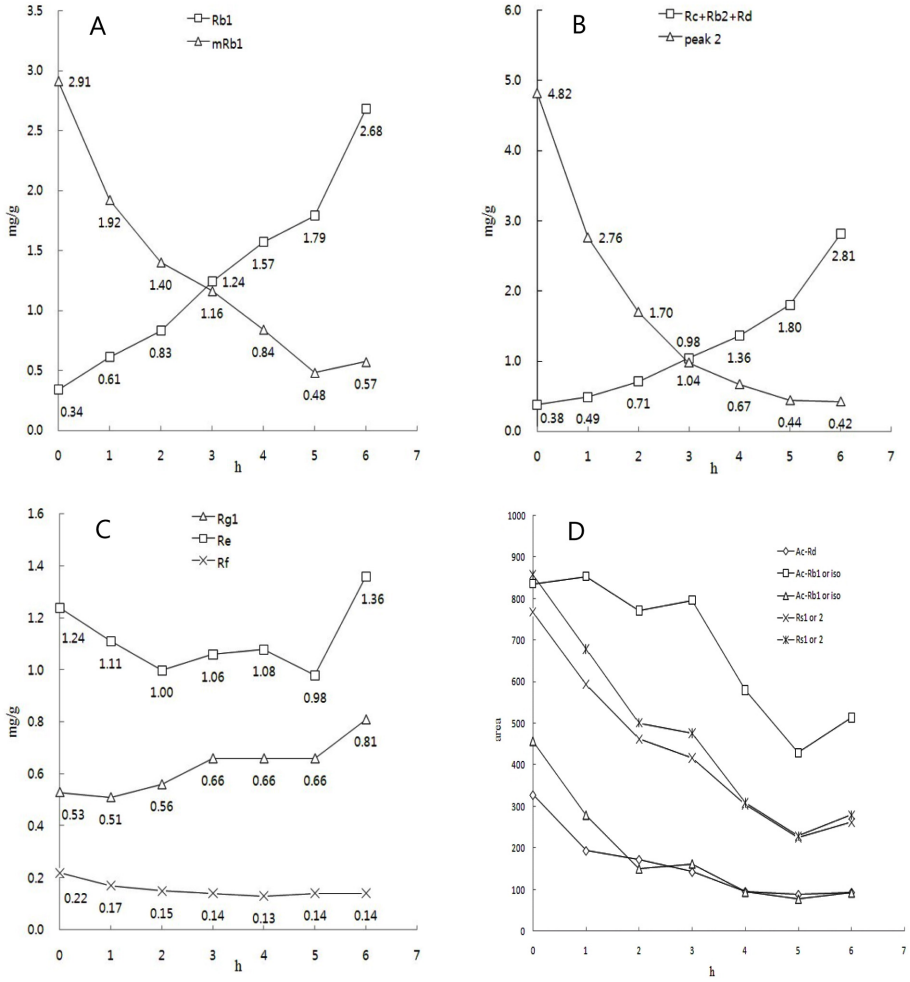

Fig. 1. Variations in the amount of individual ginsenosides by the heating time of fresh *P. ginseng* roots. All data is given by triplicate experiments. The amounts of all malonyl ginsenosides are approximated by indirect analysis method. Rb_1 and the corresponding ginsenoside mRb_1 (A), peak 2 is sum of approximated content values of mRc, mRb2, and mRd (B), the corresponding malonyl ginsenosides of protopanaxatriol ginsenosides are not found (C), the y-axis values are area of selected ion peaks of each compound (D).

Re, Rf, Rb_1, Rc, Rb_2, and Rd, each ginsenoside (10 mg) in 20 mL of citric acid (pH 3, 20 mM) was heated at 85 °C for 4 h. After cooling, the reaction mixtures were neutralized with 0.01% NaOH for chromatography to be conducted later.

Results and Discussion

Malonyl ginsenosides, which are thermally unstable, can degrade into corresponding neutral ginsenosides.[12] To study the degradation of malonyl ginsenosides into neutral ginsenosides, I performed indirect analysis applying a previously reported method[10] due to unavailable standards. The content of $mRb_1$was estimated from a regression equation by the content of $Rb_{1,6h} - Rb_{1,0h}$ (x), the peak area of $mRb_{1,0h} - mRb_{1,6h}$ (y), and 0 as a y intercept. Identification of the mRb_1 was carried out by comparing the chromatogram of an unheated (0 h) and 6-h heated samples, and also with a previous reported chromatogram obtained by a similar analytical method to ours.[13] The concentrations of mRb_1 and Rb_1 are changed depending on the heating time. Particularly, as heating time increased, the content of Rb_1 was increased, whereas mRb_1 was decreased significantly (Fig. 1A, Fig. 2). Ginsenoside Rb_1 is an important quality control marker substance together with Rg_1, but most content of Rb_1 in ginseng is not naturally occurring. The content of Rb_1 in 6-h heated fresh ginseng ($Rb_{1,6h}$: 2.68 mg/g) is higher than for unheated ($Rb_{1,0h}$: 0.34 mg/g) by a factor of 8. This result means that 87% of Rb_1 originated from mRb_1. In general, only trace level mlonyl ginsenosides were found in red ginseng due to most of the mlonyl ginsenosides degrading to neutral ginsenosides. Therefore, most Rb_1 in red ginseng is an artifact demalonylated from mRb_1 in fresh ginseng.

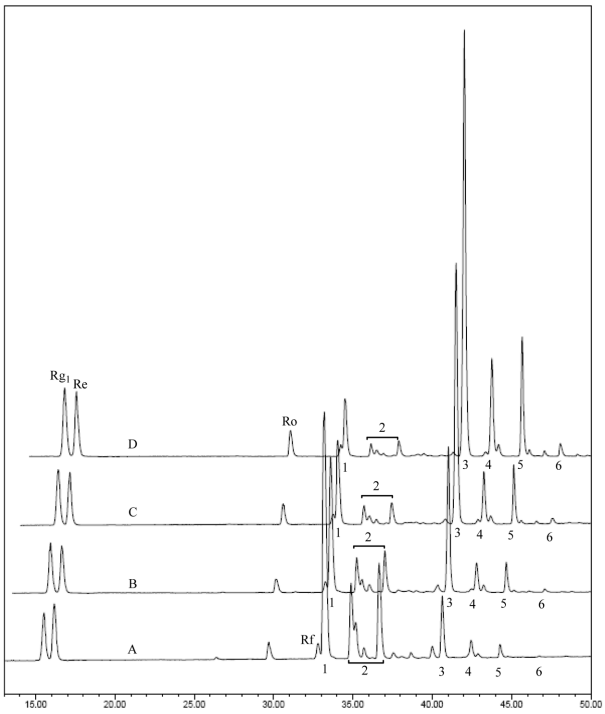

Fig. 2. HPLC/ELSD chromatograms of hourly heating of fresh ginseng: (A) 0 h, (B) 2 h, (C) 4 h, (D) 6 h heating, 1: mRb_1, 2: mixture of mRc, mRb_2, mRd, 3: Rb_1, 4: Rc, 5: Rd, and 6: Rd.

Changes in the content of protopanaxdiols such as Rc, Rb_2, and Rd are also observed. As shown in Fig. 1B, the contents of Rc, Rb_2, and Rd in 6-h heated ginseng are about 7, 8, and 9 times higher than the contents of fresh ginseng, respectively. In contrast, the total peak areas (Fig. 1B, Fig. 2) estimated as malonyl ginsenosides against neutral ginsenosides is reduced symmetrically. These results prove that most of the neutral protopanaxadiol ginsenosides Rb_1, Rc, Rb_2, and Rd in red ginseng are derived from malonyl panaxadiol ginsenoside in fresh ginseng. However, the notable changes of contents are not founded in any samples against protopanaxatriol ginsenosides Rg_1, Re, and Rf (Fig. 1C, Fig. 2). I think that these are the naturally occurring ginsenosides after considering the fact that the corresponding malonyl ginsenosides have not been found until now.

Based on a previous report, malonyl ginsenosides are converted to corresponding acetyl ginsenosides as monoacetates at the 6-hydroxyl group of the terminal glucosyl moiety of the sophorosyl unit of ginsenosides.[14] According to the results of our study with UPLC-Q-TOF-MS analysis, however, the content of acetyl ginsenosides (Ac-Rd, Ac-Rb_1, Rs_1, and Rs_2, Fig. 3) in fresh ginseng is higher than in heated ginseng samples (Fig. 1D).

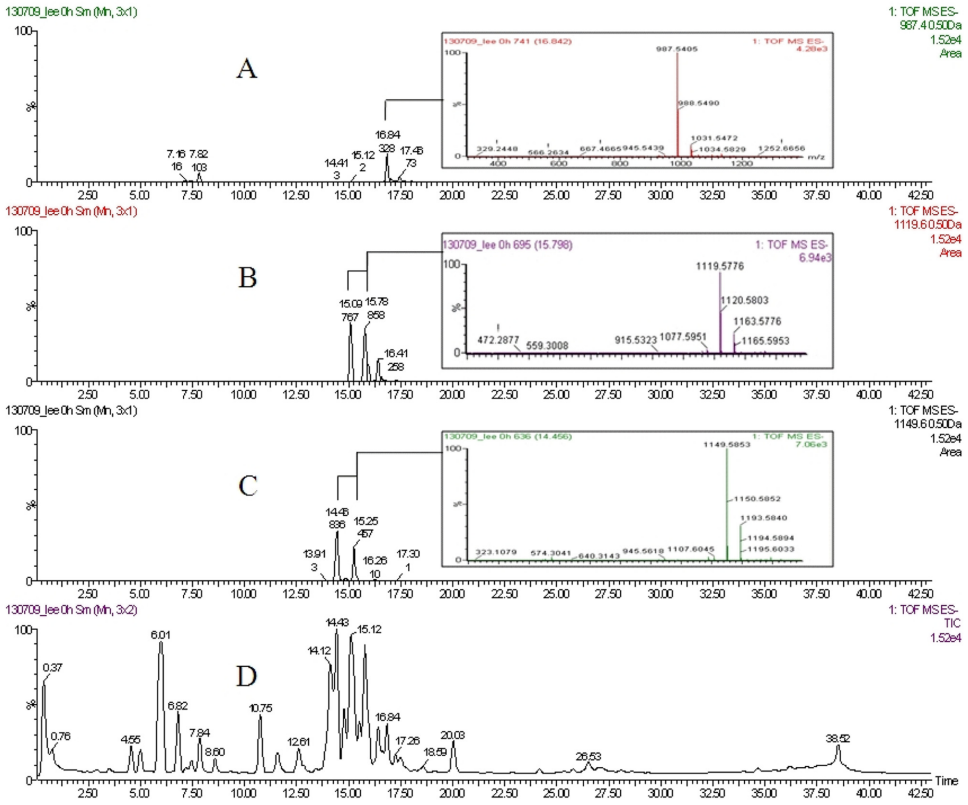

Fig. 3. The selected and total ion (D) chromatography of 0-h heated fresh *p. ginseng* roots. (A): 987.4 m/z; Ac-Rd (calcd. $[M - H]^- = 987.48$), (B) 1119.6 m/z; Ac-Rc, -Rb_2 (calcd. $[M - H]^- = 1119.60$), (C) 1149.6 m/z; Ac-Rb_1, iso (calcd. $[M - H]^- = 1149.62$).

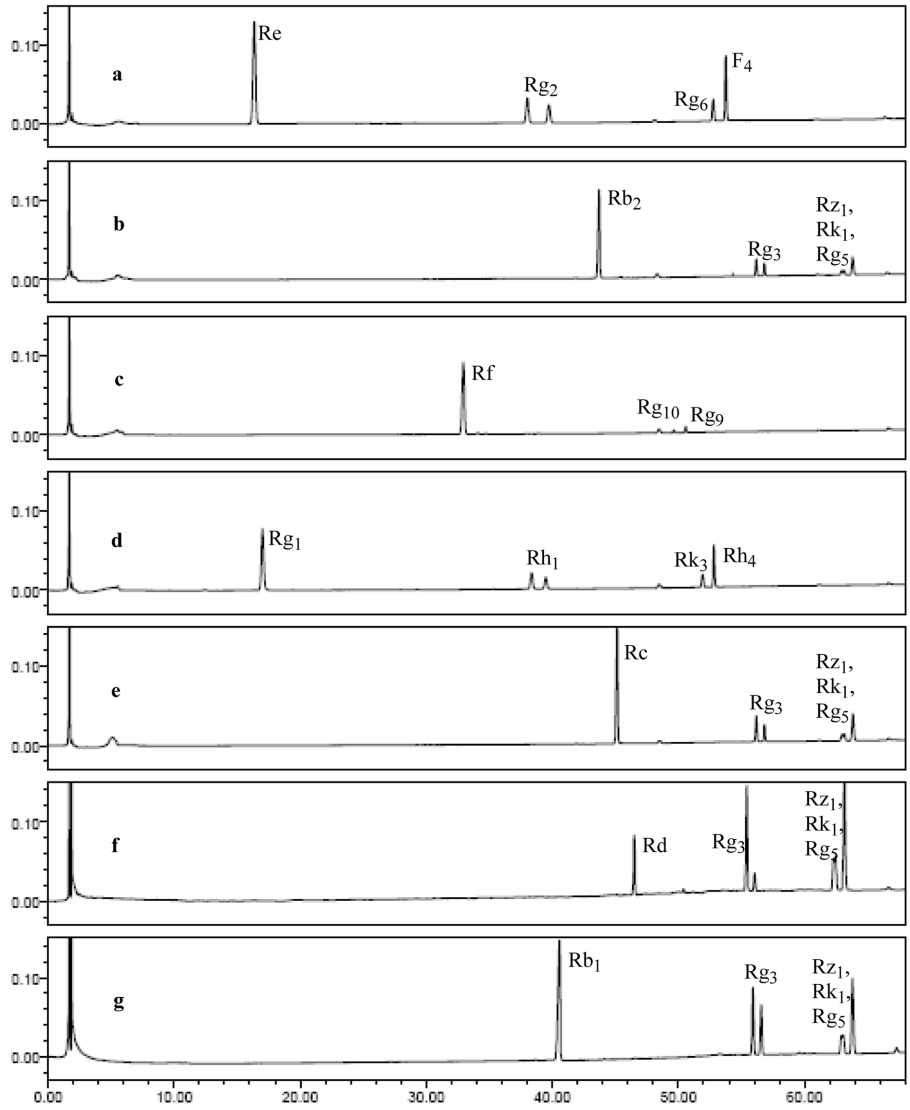

Fig. 4. Chromatography of conversion ginsenosides, which were converted from ginsenoside Re (**a**), ginsenoside Rb_2 (**b**), ginsenoside Rf (**c**), ginsenoside Rg_1 (**d**), ginsenoside Rc (**e**), ginsenoside Rd (**f**), ginsenoside Rb_1 (**g**). Each ginsenoside (10 mg) in 20 mL of citric acid (pH 3, 20 mM) was heated at 85 °C for 4 h. Specificity of each peak are confirmed by Q-TOF-Mass spectrum; Re (*m/z*: 945.5425 $[M-H]^-$), 20(S)-Rg_2 (*m/z*: 783.4976 $[M-H]^-$), 20(R)-Rg_2 (*m/z*: 783.4974 $[M-H]^-$), Rg_6 (*m/z*: 765.4805 $[M-H]^-$), F_4 (*m/z*: 765.4825 $[M-H]^-$), Rb_2 (*m/z*: 1077.5850 $[M-H]^-$), 20(S)-Rg_3 (*m/z*: 783.4890 $[M-H]^-$), 20(R)-Rg_3 (*m/z*: 783.4910 $[M-H]^-$), Rz_1 (*m/z*: 765.4871 $[M-H]^-$), Rk_1 (*m/z*: 765.4805 $[M-H]^-$), Rg_5 (*m/z*: 765.4826 $[M-H]^-$), Rf (*m/z*: 799.4908 $[M-H]^-$), Rg_1 (*m/z*: 799.4875 $[M-H]^-$), 20(S)-Rh_1 (*m/z*: 637.4412 $[M-H]^-$), 20(R)-Rh_1 (*m/z*: 637.4297 $[M-H]^-$), Rk_3 (*m/z*: 665.4265 $[M-H+HCO_2H]^-$), Rh_4 (*m/z*: 665.4235 $[M-H+HCO_2H]^-$), Rc (*m/z*: 1077.5988 $[M-H]^-$), Rd (*m/z*: 945.5500 $[M-H]^-$), Rb_1 (*m/z*: 1107.6090 $[M-H]^-$).

Therefore, I think that acetyl ginsenosides are naturally occurring constituents in fresh ginseng, not decarboxylates from malonyl ginsenosides.

To learn more about the ginsenoside conversion mechanism, we isolated protopanaxatriol (Rg_1, Re, and Rf) and protopanaxadiol (Rb_1, Rc, Rb_2, and Rd) ginsenosides as initial substances to study of conversion patterns in heated process of ginseng. These compounds, which are neutral ginsenosides in ginseng, are denatured by heating under acidic conditions. The reaction solvent to simulate with red ginseng production conditions was acidic water (citric acid 20 mM, adjusted at pH 3 with calcium carbonate). The conversion reaction was performed at 85 °C for 4 h. Citric acid as a reaction catalyst is an important organic acid element in ginseng together with malonic acid,[15] and the acidity is determind from the internal acidity of fresh ginseng. Chromatographic analysis was performed to confirm the conversion of each sample of ginsenosides. Using their chromatographic data (Fig. 4), the conversions from each ginseno-

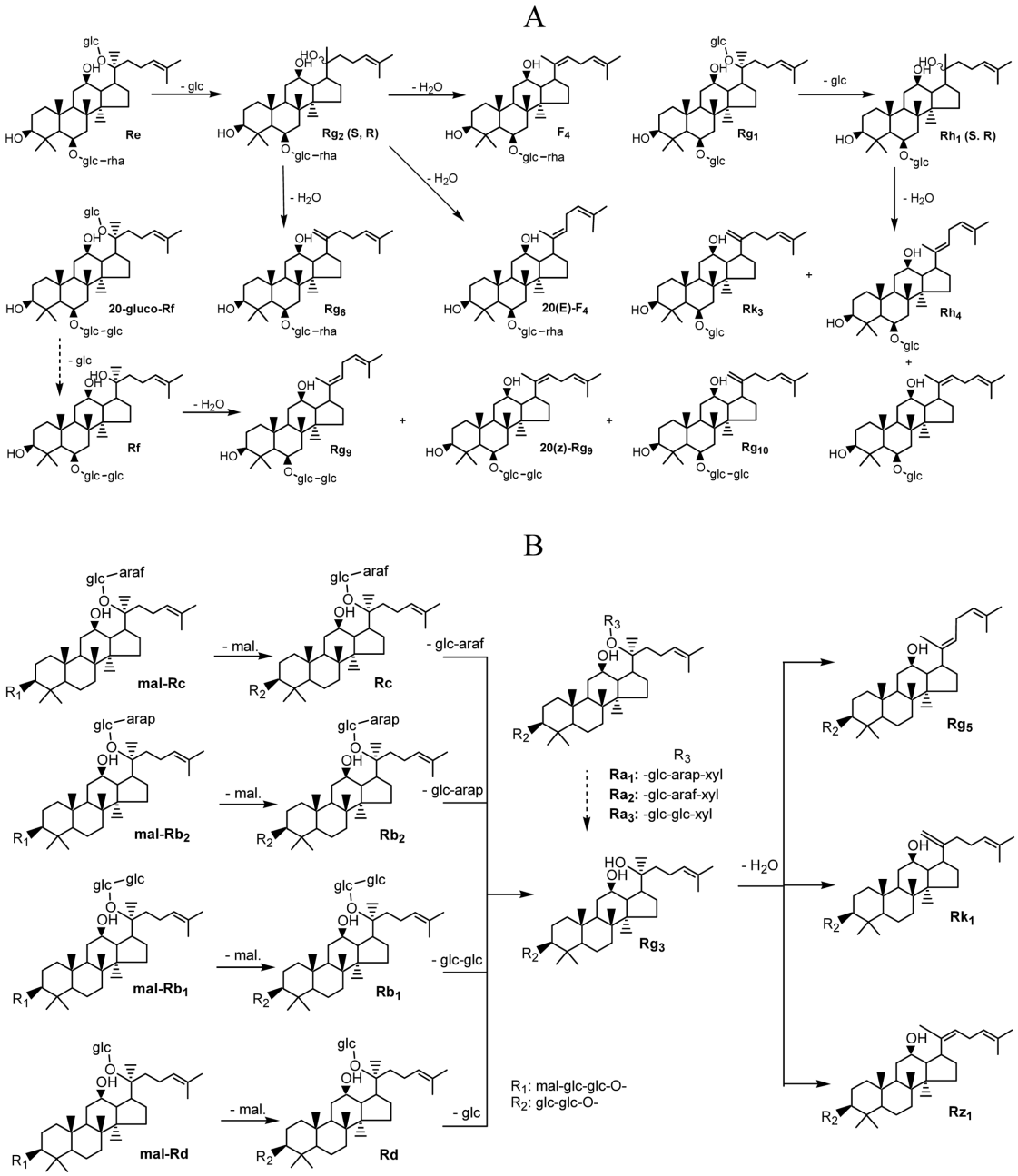

Fig. 5. Estimated conversion mechanisms of protopanaxatriol (A), protopanaxadiol (B) ginsenosides from heat-processed ginseng. –: by experiment, ----: by rule.

side were estimated as follows: $Rg_1 \rightarrow Rh_1 \rightarrow Rh_4$ and Rk_3; Re $\rightarrow Rg_2 \rightarrow F_4$, and Rg_6; Rf $\rightarrow Rg_9$, 20Z-Rg_9 and Rg_{10};[16] Rb_1, Rc, Rb_2, and Rd $\rightarrow Rg_3 \rightarrow Rg_5$, Rk_1, and Rz_1.

In the case of protopanaxatriol ginsenosides (Fig. 5A) under acidic conditions produced by citric acid, Rg_1 converts to Rh_1 by hydrolysis of the 20-glucose, and then converts to Rh_4 and Rk_3 by a dehydration reaction. However, the 20(E)-Rh_4 as an epimer of Rh_4 remains unfound. Ginsenoside Re also converts to Rg_2 by hydrolysis of the 20-glucose, and then converts to 20(E)-F_4, F_4, and Rg_6 as with the conversion process of Rg_1. Ginsenoside Rf with no 20-glucose converts to Rg_9 and Rg_{10} by the dehydration of 20-OH. When panaxadiol ginsenoside (Fig. 5B) is exposed to acidic conditions produced by citric acid, all of the Rb_1, Rb_2, Rc, and Rd are converted to Rg_3 by hydrolysis of the 20-glycoside. Therefore, the more the ginseng is heated, the higher the

content of Rg_3. These results explain that the contents of conversion ginsenosides such as Rg_2, Rh_1, and Rg_3 progressively increase, and the contents of natural ginsenosides such as Rg_1, Re, Rb_1, Rc, and Rd progressively decrease in heat-processed red ginseng production.[16] I believe that Rg_3 is ultimately converted to ginsenosides Rg_5, Rk_1, and Rz_1, because Rg_3 does not increase beyond a certain concentration.[17] According to these results and the references,[14,18] I infer that the following 3 rules exist for conversion of ginsenosides under acidic conditions: the ether bond should be stable at positions 3 and 6, the ether bond between sugars is stable, and the ether bond is unstable at position 20 in the dammarane ginsenoside.

Acknowledgements

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