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Efficient Thermal Deglycosylation of Ginsenoside Rd and Its Contribution to the Improved Anticancer Activity of Ginseng

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ABSTRACT: The root of ginseng is a famous functional food and a herbal medicine. Research into the development of a method for increasing the pharmaceutical effect of ginseng by conversion of ginsenosides, the major active components of ginseng, by high-temperature and high-pressure thermal processing has been conducted. However, changes in the structures of each ginsenoside by heat processing and their contributions to anticancer activity have not yet been fully elucidated. Here, we investigated whether anticancer activity of ginsenosides, such as Rb_1, Rb_2, Rc, Rd, and Re, was associated with changes in the structures of each ginsenoside by heat processing in human stomach cancer AGS cells. Upon heat processing at 120 °C, most peaks of ginsenosides Rb_1, Rb_2, Rc, and Rd disappeared and the contents of less-polar ginsenosides 20(*S*,*R*)-Rg_3, Rk_1, and Rg_5 were newly detected. From the quantitative analysis of ginsenosides, the generated amounts of less-polar ginsenosides were the highest after heat processing of ginsenoside Rd. After heat processing, the diol-type ginsenosides Rb_1, Rb_2, Rc, and Rd gained significant antiproliferative activity. In particular, ginsenoside Rd induced the strongest cell death among the diol-type ginsenosides, whereas the triol-type gisenoside Re showed weak antiproliferative activity. Ginsenoside Rd-induced cell death was associated with caspase-dependent apoptosis. Taken together, these results demonstrate that deglycosylation of Rd contributes to improved anticancer activity of ginseng and provide new insight on the mechanism of increased anticancer effects of ginsenosides upon heat processing.

KEYWORDS: *Ginsenoside, Panax ginseng, Maillard reaction, free radical, heat processing*

INTRODUCTION

The root of ginseng, *Panax ginseng* C.A. Meyer (Araliaceae), is a famous functional food and a herbal medicine in the Orient. *P. ginseng* is first heat-processed to improve its medicinal efficacies, such as its antioxidant and anticancer activities.[1−5] In Korea, *P. ginseng* is harvested after 4−6 years of cultivation and is classified into three types depending upon how it is processed. White ginseng is produced by drying the ginseng root. Red ginseng is made by steaming the ginseng root at 98−100 °C (Figure 1A).[6] Red ginseng is more common as a medicinal herb than white ginseng in Asian countries, because steaming induces changes in the chemical constituents and enhances the biological activities of ginseng.[6−8] A novel heat-processing method of autoclaving ginseng at a higher temperature than that used to produce red ginseng was recently developed to achieve an even stronger activity than that of red ginseng, and this ginseng product was termed heat-processed ginseng (HPG; Figure 1A).[9,10]

These plants in *Panax* species contain dammarane-based saponin in common with 1−4 saccharide(s) attached to a dammarane backbone, unlike other plants.[3,6] In particular, saponins are found at high concentrations in ginseng, including ginsenosides Rb_1, Rb_2, Rc, Rd, and Re. Previous studies have indicated that several ginsenosides derived from *Panax* species have anticancer activity against cancer cells by triggering apoptotic cell death.[4,11,12] Anticancer activities of ginsenosides are greatly different in types and intensities depending upon their structures.[13] Hasegawa et al. also proposed that the deglycosylated ginseng saponin metabolite M1 was esterified with fatty acids in the liver, which resulted in longer permanence in the body and exhibited antitumor activities, and concluded that ginsenoside is a pro-drug activated in the body by deglycosylation and esterification.[14] In addition to these findings, we have recently identified that structural changes of certain ginsenosides by heat processing are significantly related to their improved anticancer activities.[15]

In our recent research on the triol-type ginsenoside Re, the sugar moieties at carbon 20 were deglycosylated by heat processing, and they were gradually changed into 20(*S*)-Rg_2, 20(*R*)-Rg_2, Rg_6, and F_4, which have stronger anticancer activities than that of the mother compound itself.[15] However, structural changes of other major ginsenosides by heat processing and their contribution to anticancer activity have not yet been elucidated. Therefore, active ginsenosides with enhanced anticancer activities and their mechanisms of action were investigated in this study to elucidate scientific evidence underlying the heat processing of *P. ginseng*.

MATERIALS AND METHODS

Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY). The EZ-Cytox enhanced cell viability assay kit was purchased from ITSBIO (Seoul, Korea). Ginsenosides Rb_1, Rb_2, Rc, Rd, Re, 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, Rg_5 20(*S*)-Rg_2, 20(*R*)-Rg_2, Rg_6, and F_4 were purchased from Ambo Institute (Seoul, Korea). The water and acetonitrile used were of high-performance liquid chromatography (HPLC) grade from Fisher

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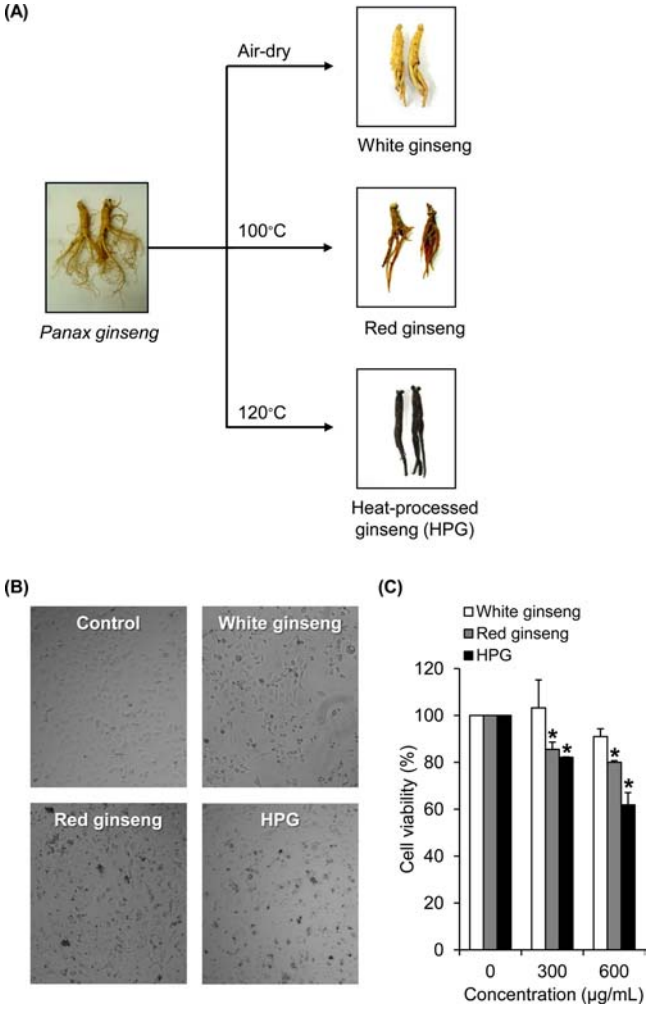

Figure 1. Classification of *P. ginseng* products and their anticancer activities. (A) Classification of *P. ginseng* products by heat-processing methods. (B) Morphological changes were confirmed using phase-contrast microscopy. (C) Changes in effects of ginseng upon heat processing on AGS cell proliferation. Cells were treated with ginseng products at different concentrations (0, 300, and 600 μg/mL) for 24 h. Relative cell proliferation was measured by the CCK-8 assay. Each value represents the mean ± standard deviation (SD) of three independent experiments. (∗) $p < 0.05$ compared to the not treated value.

Scientific (Pittsburgh, PA), and glacial acetic acid was of analytical grade from Sigma-Aldrich (St. Louis, MO).

Preparation of Ginseng Extracts. The four-year-old fresh ginseng (*P. ginseng*) was purchased from a local ginseng market in Seoul, Korea. White ginseng was produced by drying 100 g of fresh ginseng at 50 °C for 3 days. Red ginseng was made by steaming white ginseng at 100 °C for 3 h using an autoclave (gauge pressure of 0.03 MPa) and drying at 50 °C for 3 days. HPG was made by steaming white ginseng at 120 °C for 3 h using an autoclave (gauge pressure of 0.11 MPa) and drying at 50 °C for 3 days.[16] The three ginsengs were ground to pass an 80-mesh sieve, extracted under reflux with MeOH 3 times at 70 °C for 2 h, and filtered through filter paper (Advantec, Tokyo, Japan), and the solvent was evaporated *in vacuo* to give a MeOH extract with a yield of about 20%, by weight, of the original ginseng powder.

Heat-Processing Model Experiment Using Ginsenosides. The reaction was conducted in 2.0 mL of polypropylene centrifuge tubes, which can be sterilized by autoclaving. The centrifuge tubes containing 2 mg of each ginsenoside (Rb_1, Rb_2, Rc, Rd, and Re) dissolved in 200 μL of distilled water were steamed at 120 °C for 3 h using an autoclave (gauge pressure of 0.11 MPa). After drying at 50 °C for 3 days, untreated and heat-processed ginsenosides at 120 °C were prepared.

Analysis of Ginsenosides. Ginsenosides were identified by the comparison of retention times and molecular weights to those of standard samples. An analytical reversed-phase HPLC system was composed of a solvent degasser (Agilent, G1322A), a binary pump (Agilent, G1312C), an autosampler (Agilent, G1329B), and a model 380 evaporative light scattering detector (ELSD) (Agilent). ELSD conditions were optimized to achieve maximum sensitivity: temperature of the nebulizer was set for 50 °C, and N_2 was used as the nebulizing gas at a pressure of 2.0 bar. The Phenomenex Luna C18 column (150 × 4.6 mm, 5 μm) was used, and the mobile phase consisted of a binary gradient of solvent A (15:80:5 acetonitrile/water/5% acetic acid in water) and solvent B (80:20 acetonitrile/water) at a flow rate of 1.0 mL/min. The gradient flow program was as follows: initial, 0% B; 6 min, 30% B; 18 min, 50% B; 30 min, 100% B; 37 min, 100% B; and 42 min, 0% B. Low-resolution electrospray ionization mass spectrometry (ESI−MS) data were measured with an Agilent Technologies VS/Agilent 1100 system (Santa Clara, CA). The amounts of ginsenosides in samples were quantified as reported previously.[12] The standard solutions containing 1−50 μg of each ginsenoside were injected into HPLC, and all calibration curves showed good linearity ($R^2 > 0.995$). The analysis was repeated 2 times for the verification of repeatability.

Antiproliferative Effect on Gastric Cancer Cells. The human gastric cancer AGS cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in RPMI 1640 medium (Cellgro, Manassas, VA) supplemented with 10% FBS (Gibco BRL, Gaithersburg, MD), 100 units/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 °C in a humidified atmosphere with 5% CO_2. AGS cells were treated with different concentrations of compounds for 24 h, and cell proliferation was measured using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) according to the recommendations of the manufacturer.

Western Blotting Analysis. AGS cells were grown in 6-well plates and treated with the indicated concentration of compounds for 24 h. Whole-cell extracts were then prepared according to the instructions of the manufacturer using RIPA buffer (Cell Signaling, Beverly, MA) supplemented with 1× protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride (PMSF). Proteins (whole-cell extracts, 30 μg/lane) were separated by electrophoresis in a precast 4−15% Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA) blotted onto polyvinylidene difluoride (PVDF) transfer membranes and analyzed with epitope-specific primary and secondary antibodies. Bound antibodies were visualized using ECL Advance Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, U.K.) and a LAS 4000 imaging system (Fujifilm, Japan).

Statistical Analysis. Statistical significance was determined through analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment. p values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Changes in the Chemical Composition and Anticancer Activity of Ginseng by Heat Processing. To estimate the anticancer activities of ginseng products, AGS cells were treated with each of their extracts at the indicated concentrations for 24 h and then their proliferation was examined. As shown in Figure 1B, there were no morphological changes in white-ginseng-treated cells; however, lower confluency and more debris of cells were observed in red-ginseng- or HPG-treated cells. More specifically, treatments of HPG at 300 and 600 μg/mL triggered cell death by 17.8 and 38.2%, respectively (Figure 1C). HPG had the most powerful anticancer activity among the ginsengs against AGS cells.

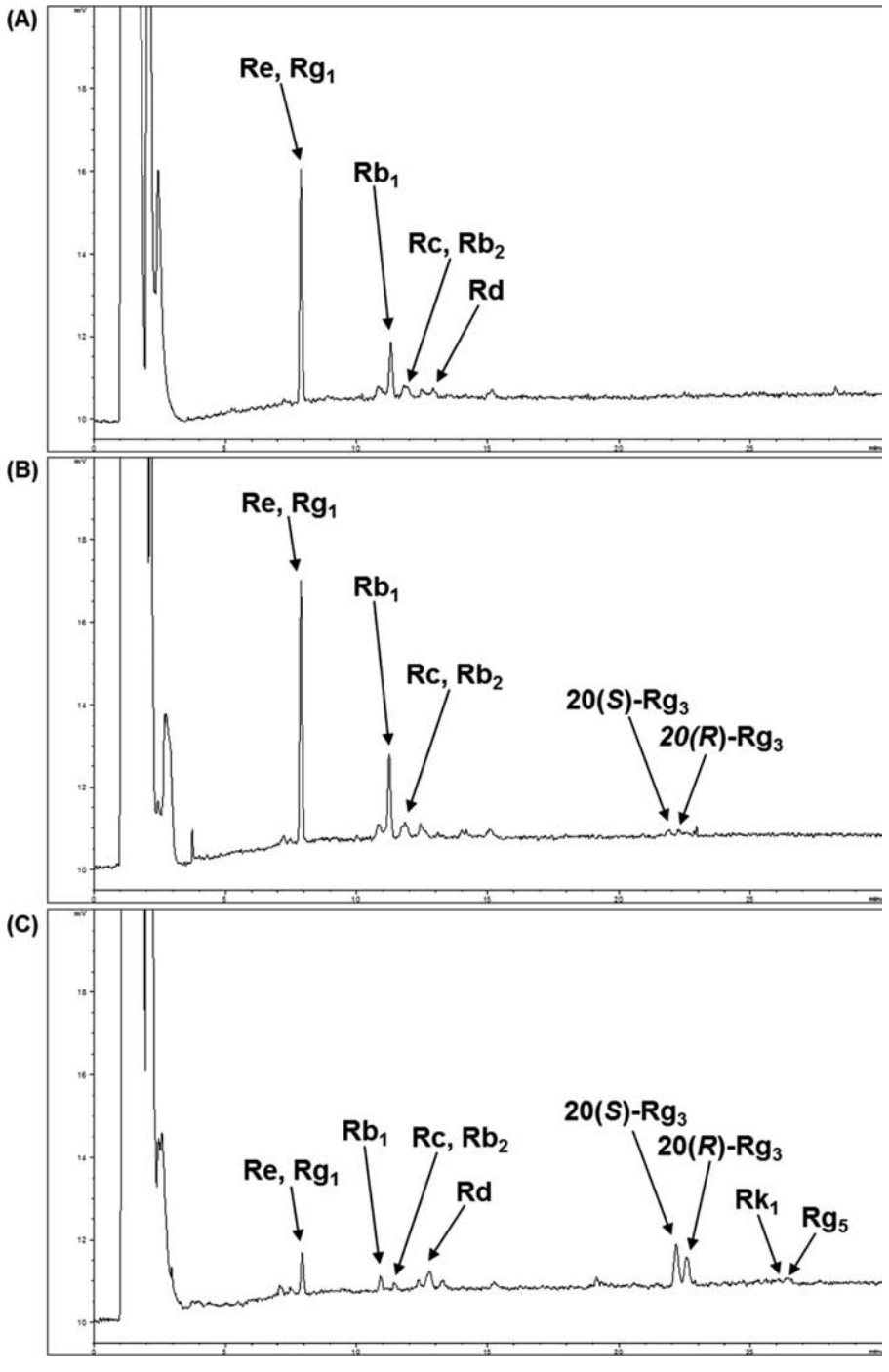

Figure 2. Comparison in the HPLC chromatograms of (A) white ginseng, (B) red ginseng, and (C) HPG.

The HPLC chromatogram of each prepared ginseng extract is illustrated in Figure 2. White ginseng shows typical ginsenosides consisting of Re, Rb_1, Rc, Rb_2, and Rd (Figure 2A). In the case of red ginseng, the contents of ginsenosides Re, Rb_1, Rc, and Rb_2 are slightly increased because a small portion of these ginsenosides usually exists as a malonylated form, which is readily demalonylated by heating. In addition, the peak of ginsenoside Rd disappeared and the peaks of ginsenoside 20(*S*,*R*)-Rg_3 were newly detected in red ginseng (Figure 2B). After heat processing at 120 °C, the major ginsenosides Re, Rb_1, Rc, and Rb_2 contained in white ginseng were greatly decreased but the contents of ginsenoside 20(*S*,*R*)-Rg_3 were increased. Interestingly, the peak of ginsenoside Rd was slightly increased than that of white ginseng, and the peaks of Rk_1 and Rg_5 were newly detected in HPG (Figure 2C).

In this study, HPG showed a stronger antiproliferative effect against AGS cells than white ginseng and red ginseng. This finding suggests that heat processing provides ginseng strong anticancer activity and changes in ginsenoside composition.

Changes in the Chemical Structure and Anticancer Activity of Ginsenosides by Heat Processing. We have conducted several heat-processing model experiments using ginsenosides Rb_1, Rb_2, Rc, Rd, and Re to verify the changes in the structures of each ginsenoside through heat processing and their contributions to the increased anticancer activity, which have not been elucidated. As shown in the HPLC chromato-

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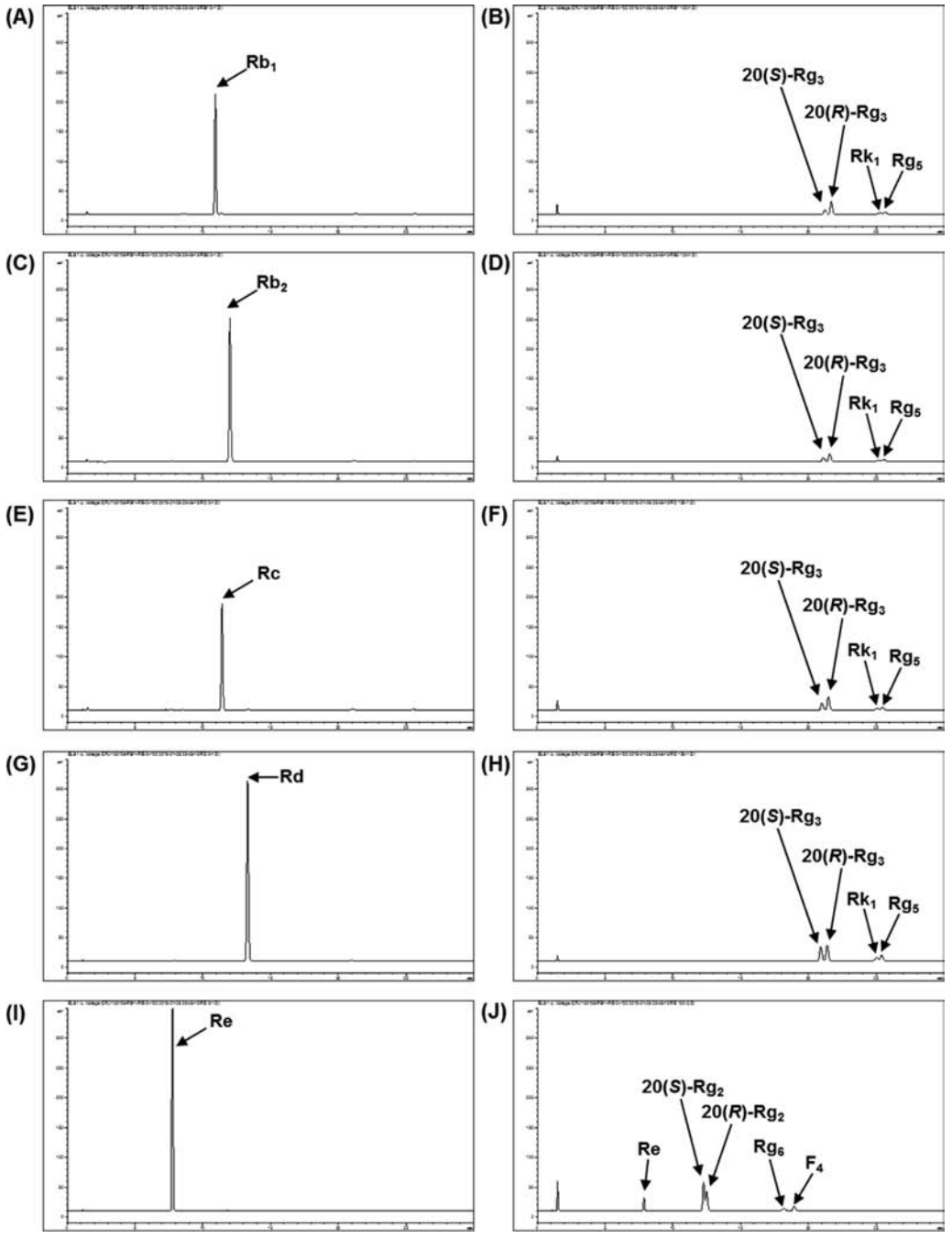

Figure 3. Comparison in the HPLC chromatograms of ginsenosides by heat processing at 120 °C. (A) HPLC chromatogram of ginsenoside Rb_1 before heat processing. (B) HPLC chromatogram of ginsenoside Rb_1 after heat processing. (C) HPLC chromatogram of ginsenoside Rb_2 before heat processing. (D) HPLC chromatogram of ginsenoside Rb_2 after heat processing. (E) HPLC chromatogram of ginsenoside Rc before heat processing. (F) HPLC chromatogram of ginsenoside Rc after heat processing. (G) HPLC chromatogram of ginsenoside Rd before heat processing. (H) HPLC chromatogram of ginsenoside Rd after heat processing. (I) HPLC chromatogram of ginsenoside Re before heat processing. (J) HPLC chromatogram of ginsenoside Re after heat processing.

grams of the diol-type ginsenosides (Figure 3), ginsenosides Rb_1, Rb_2, Rc, and Rd were detected at about 11.0, 12.0, 11.4, and 13.2 min, respectively (panels A, C, E, and G of Figure 3). However, upon heat processing at 120 °C, most of these peaks disappeared and the contents of the less-polar ginsenosides 20(*S*,*R*)-Rg_3, Rk_1, and Rg_5 were newly detected (panels B, D, F, and H of Figure 3). A quantitative analysis of ginsenosides revealed that the generated amounts of less-polar ginsenosides (979.3 μg/mg) after heat processing of ginsenoside Rd were 229, 309, and 161% higher than those of ginsenosides Rb_1, Rb_2, and Rc, respectively (Table 1). In the case of triol-type ginsenoside, ginsenoside Re was detected at about 7.9 min and its peak was greatly decreased upon heat processing at 120 °C (panels I and J of Figure 3). As a result, ginsenosides in white ginseng were gradually deglycosylated and transformed into less-polar ginsenosides during heat processing (Figure 4).

To determine changes in the anticancer activity of ginsenosides after heat processing, AGS cells were treated with each ginsenoside at the indicated concentrations for 24 h and then their proliferation was examined. Ginsenosides Rb_1, Rb_2, and

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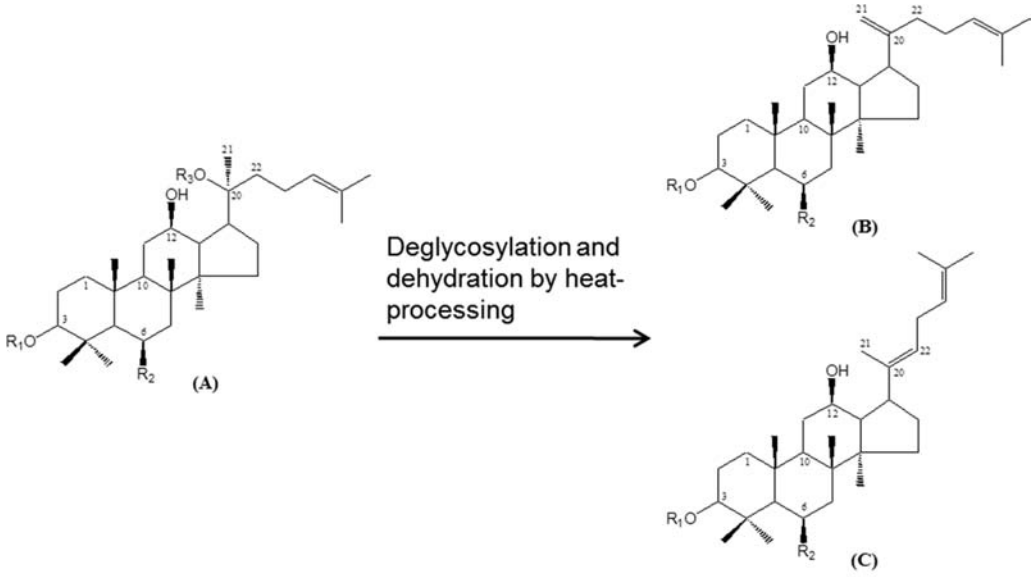
Table 1. Comparisons in the Generated Amounts of Less-Polar Ginsenosides after Heat Processing of Diol-Type Ginsenosides (μg/mg)

	Rb_1	Rb_2	Rc	Rd
20(*S*)-Rg_3	90.7	85.2	173.1	365.1
20(*R*)-Rg_3	248.3	160.8	309.5	377.4
Rg_5	37.6	29.6	55.8	98.6
Rk_1	49.3	41.1	68.5	138.2

Rc showed very similar effects with no cytotoxicity before heat processing but significant cytotoxicity (IC_{50} values of 27.7, 28.9, and 28.3 μg/mL, respectively) after heat processing (panels A–C of Figure 5). Ginsenoside Rd also inhibited cell proliferation (IC_{50} value of 20.1 μg/mL) after heat processing; however, unlike Rb_1, Rb_2, and Rc, definite cell death was induced by 100 μg/mL of Rd without heat processing (Figure 5D). On the other hand, the triol-type ginsenoside Re induced less cell death (IC_{50} value of 91.8 μg/mL) than those of diol-type ginsenosides and produced weak effects at high concentrations after heat processing (Figure 5E). Because diol-type ginsenosides gradually transform into 20(*S*,*R*)-Rg_3 and Rk_1/Rg_5 mixtures, we next investigated whether the mixtures are responsible for inducing cell death. In the comparison of anticancer activities of active ginsenoside fractions from the heat-processed ginsenoside Rd (Figure 5F), the 20(*S*,*R*)-Rg_3 fraction showed a stronger antiproliferative effect than the Rg_5/Rk_1 fraction (IC_{50} value of 23.6 versus 42.9 μg/mL).

On the basis of the result that ginsenoside Rd showed the strongest antiproliferative effect among the diol-type ginsenosides, we focused on ginsenoside Rd and examined whether its effect is involved in apoptotic cell death. As shown in Figure 6, exposure to the heat-processed ginsenoside Rd up to 30 μg/mL for 24 h induced the cleavage of PARP as well as caspase-3 and caspase-8 in a dose-dependent manner. Meanwhile, ginsenoside Rd did not cleave caspase-9. These results suggest that



Structure	Group	Ginsenoside	R1	R2	R3
(A)	PPD	Rb_1	-Glc-Glc	-H	-Glc-Glc
		Rb_2	-Glc-Glc	-H	-Glc-Ara(p)
		Rc	-Glc-Glc	-H	-Glc-Ara(f)
		Rd	-Glc-Glc	-H	-Glc
		20(*S*,*R*)-Rg_3	-Glc-Glc	-H	-H
	PPT	Re	-H	-OGlc-Rha	-Glc
		20(*S*,*R*)-Rg_2	-H	-OGlc-Rha	-H
(B)	DHPPD-I	Rk_1	-Glc-Glc	-H	
	DHPPT-I	Rg_6	-H	-OGlc-Rha	
(C)	DHPPD-II	Rg_5	-Glc-Glc	-H	
	DHPPT-II	F_4	-H	-OGlc-Rha	

Figure 4. Changes in chemical structures of ginsenosides during heat processing. −Glc, D-glucopyranosyl; −Rha, L-rhamnopyranosyl; −Ara(f), L-arabinofuranosyl; −Ara(p), L-arabinopyranosyl; PPD, protopanaxadiol; PPT, protopanaxatriol; DHPPD, dehydroprotopanaxadiol; and DHPPT, dehydroprotopanaxatriol.

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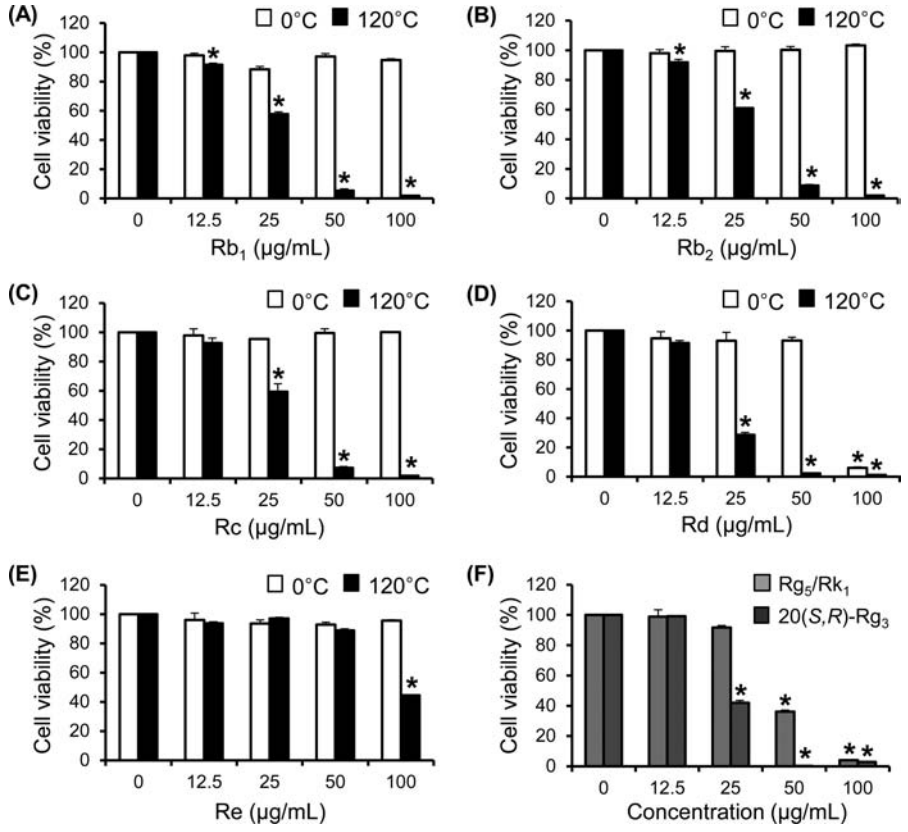

Figure 5. Changes in effects of ginsenosides upon heat processing on AGS cell proliferation. (A) Cells were treated with ginsenoside Rb_1 with or without heat processing at different concentrations (12.5, 25, 50, and 100 μg/mL) for 24 h. (B) Cells were treated with ginsenoside Rb_2 with or without heat processing at different concentrations (12.5, 25, 50, and 100 μg/mL) for 24 h. (C) Cells were treated with ginsenoside Rc with or without heat processing at different concentrations (12.5, 25, 50, and 100 μg/mL) for 24 h. (D) Cells were treated with ginsenoside Rd with or without heat processing at different concentrations (12.5, 25, 50, and 100 μg/mL) for 24 h. (E) Cells were treated with ginsenoside Re with or without heat processing at different concentrations (12.5, 25, 50, and 100 μg/mL) for 24 h. (F) Cells were treated with ginsenosides 20(*S*,*R*)-Rg_3 or Rg_5/Rk_1 at different concentrations (12.5, 25, 50, and 100 μg/mL) for 24 h. Relative cell proliferation was measured by the CCK-8 assay. Each value represents the mean ± SD of three independent experiments. (*) $p < 0.05$ compared to the not treated value.

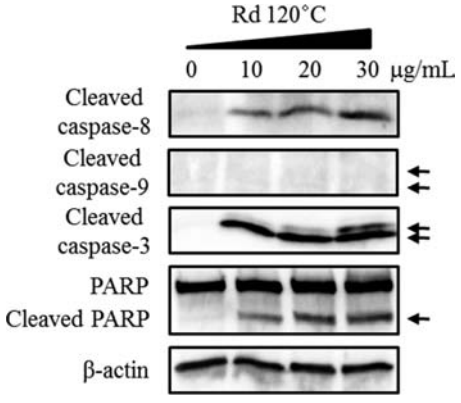

Figure 6. Effect of heat-processed ginsenoside Rd on pro-apoptotic proteins in AGS cells. Results of the western blot show the levels of cleaved caspase-8 (18 kDa), Bcl-2 (26 kDa), Bax (20.5 kDa), cleaved caspase-9 (35 and 37 kDa), cleaved caspase-3 (17 and 19 kDa), PARP (116 kDa), and cleaved PARP (85 kDa) in AGS cells treated with heat-processed ginsenoside Re at different concentrations (40, 80, and 160 μg/mL) for 24 h. A total of 30 μg of each protein were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). GAPDH (37 kDa) was used as an internal control.

deglycosylation of ginsenoside Rd and its transformation into 20(*S*,*R*)-Rg_3 by heat processing enhances its anticancer activity through induction of extrinsic apoptotic signaling in AGS cells.

Taken together, after heat processing, ginsenosides, especially the diol-type ginsenosides Rb_1, Rb_2, Rc, and Rd, significantly inhibited AGS cell proliferation. In contrast to the triol-type ginsenoside Re, these diol-type ginsenosides were converted to less-polar ginsenosides 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 by heat processing. Among the diol-type ginsenosides, ginsenoside Rd was the most easily deglycosylated, causing increased anticancer activity. Mechanistically, we found that the efficient transformation of ginsenoside Rd into less-polar ginsenosides 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 by heat processing induced apoptosis via the extrinsic pathway in AGS cells.

In conclusion, research into the development of a method for increasing the pharmaceutical effect of ginseng by conversion of ginsenosides, which are the major active components of ginseng, by high-temperature and high-pressure thermal processing has been conducted. In this study, we demonstrated for the first time that efficient thermal deglycosylation of ginsenoside Rd enhances the anticancer activity of ginseng and provides new insight on the mechanism of increased anticancer effects of ginsenosides upon heat processing.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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