Increase in the Free Radical Scavenging Activity of Ginseng by Heat-Processing

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To investigate whether or not the radical scavenging activity of ginseng is enhanced by heat processing, we evaluated the scavenging effects of white ginseng (WG), red ginseng (RG, steamed ginseng at 98—100 °C) and sun ginseng (SG, steamed ginseng at 120 °C) on nitric oxide, superoxide (O_2^-), hydroxyl (·OH) radicals and peroxynitrite ($ONOO^-$). Heat-treated ginseng (RG and SG) showed better O_2^-, $ONOO^-$ and ·OH-scavenging activities than WG. In particular, the radical scavenging activities of SG were stronger than those of RG. Furthermore, we evaluated the radical scavenging activities of maltol, salicylic acid, vanillic acid and *p*-coumaric acid, known as principal antioxidant components of ginseng, in WG, RG and SG, and also investigated their contents. Of the tested compounds, maltol, vanillic acid and *p*-coumaric acid exhibited $ONOO^-$-scavenging activity. In addition, maltol and *p*-coumaric acid showed strong ·OH-scavenging activity. Moreover, the content of maltol was remarkably increased in a temperature-dependent manner by heat processing, implying that maltol was closely related to the radical scavenging activity of heat-processed ginseng. These findings indicate that SG may act as a free radical scavenger and protect against damage caused by oxidative stress related with these radicals.

Key words sun ginseng; radical scavenging; nitric oxide (NO); superoxide (O_2^-); peroxynitrite ($ONOO^-$); hydroxyl (·OH); maltol

Reactive oxygen metabolites, including free radicals such as nitric oxide (NO), superoxide anion (O_2^-), hydroxyl (·OH) radicals and peroxynitrite ($ONOO^-$), are toxic and play an important role in tissue injury.[1,2] O_2^- reacts rapidly with NO to produce the more toxic $ONOO^-$. In addition, $ONOO^-$ and its decomposition product, ·OH, contribute to antioxidant depletion, alterations of protein structure and oxidative damage observed in human diseases.[3—5] Thus, it is important to find safe and effective scavengers of these oxygen radicals for prevention and treatment of oxidative stress-related diseases.

Panax ginseng C. A. MEYER is one of the most widely used herbal medicines in the Orient. It has a wide range of pharmacological and physiological actions, such as antiaging, immunoenhancement, antistress and antitumor.[6—8] Of the two kinds of ginseng, white ginseng (WG), is air-dried ginseng, and red ginseng (RG) is produced by steaming raw ginseng at 98—100 °C for 2—3 h. RG is reportedly more pharmacologically active than WG. These improved biological activities of ginseng result from changes in the chemical constituents that occur during steaming treatment. Ginseng saponins, referred to as ginsenosides, are believed to play a pharmacologically important role. Several investigators have reported new ginsenosides from RG that are not usually found in WG.[9,10] Recently, a method which can enhance the yield of these RG specific ginsenosides by steaming ginseng at a temperature higher than RG has been developed.[11] This heat-processed ginseng, termed sun ginseng (SG), has been reported to have more potent pharmacological activities, such as vasorelaxation, antioxidant and antitumor activities.[12,13] In our preliminary study, SG also showed better NO-scavenging activity than conventional WG and RG. However, almost all ginsenosides had no effects on radical scavenging activity (data not shown). It is insufficient to explain the various pharmacological effects of ginseng with only ginsenosides, especially antioxidant activity related to radical scavenging.

Therefore, in this study, we focused on phenolic compounds in ginseng, such as maltol, salicylic acid, vanillic acid and *p*-coumaric acid, known as principal antioxidant components of ginseng, to investigate the radical scavenging activity of SG. We examined the NO, O_2^-, $ONOO^-$ and ·OH-scavenging activities of WG, RG and SG. Furthermore, we also studied the radical scavenging activities of maltol, salicylic acid, vanillic acid and *p*-coumaric acid and the contents of these 4 phenolic compounds in WG, RG and SG.

MATERIALS AND METHODS

Reagents Salicylic acid, *p*-coumaric acid, vanillic acid, maltol, sodium nitroprusside (SNP), nitro blue tetrazolium (NBT), β-nicotineamide adenine dinucleotide disodium salt reduced form (β-NADH), DL-penicillamine and L(+)-ascorbic acid were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane, *n*-eicosane, phenazine methosulfate (PMS), 3-morpholinosydnomine (SIN-1), dihydrorhodamine 123 (DHR 123) and curcumin were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) was obtained from Dojindo (Kumamoto, Japan). The other chemicals and reagents used were of high quality and obtained from commercial sources.

Preparations of WG, RG and SG Four-year-old fresh ginseng (*Panax ginseng* C. A. MEYER) was purchased from a local ginseng market in Seoul. WG powder was produced by drying fresh ginseng at 50 °C for 3 d and then powdered finely. RG or SG was made by autoclaving WG at 100 or 120 °C for 3 h, respectively. Each ginseng powder was homogenized for 2 min using a Polytron (Kinematica, Luzern, Switzerland) with 50% EtOH as an extraction solution. After homogenization, all samples were ultrasonificated for 30 min and filtered through filter paper (Advantec, Tokyo, Japan).

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The filtrates were vacuum evaporated to give an extract with a yield of about 20%.

Preparation of Phenolic Fraction Free and esterified phenolic compounds were extracted and fractionated as described by Jung *et al.*[14] with a slight modification. Two grams of ginseng extracts were dissolved with 20 ml of water. Aqueous suspensions were adjusted to pH 2 with 6 N HCl and centrifuged to separate a cloudy precipitate. The clear supernatant was extracted five times with hexane by manually shaking at a hexane to water ratio of 1 : 1 to remove free fatty acids and other lipid contaminants. The free phenolic fraction was extracted six times with diethyl ether–ethyl acetate (DE–EA, 1 : 1, v/v). On the other hand, the esterified phenolic fraction was prepared with the hydrolyzation of aqueous residue. The residue was hydrolyzed with 30 ml of 3 M NaOH and stirred overnight at room temperature (about 16 h) using a magnetic stirrer. The solution was then adjusted to pH 2 and liberated phenolic acids were extracted with DE–EA (1 : 1, v/v). The free and esterified phenolic fractions were evaporated to dryness under a vacuum at 30 °C and the dry residues were transferred into vials containing a known amount of internal standard (*n*-eicosane) using DE–EA as a solvent and dried under nitrogen at room temperature before silylation.

Analysis of Phenolic Fraction According to the method of Jung *et al.*[14] with a slight modification, each DE–EA extract was trimethylsilated (TMS) before the GC-MS analysis. The TMS derivatives of the phenolic compounds were separated and analyzed on a Hewlett Packard HP6890 (Agilent Technologies, Palo Alto, CA, U.S.A.) gas chromatograph coupled to a JEOL JMS-GC mate II (Jeol Ltd., Tokyo, Japan). The GC was equipped with an autosampler and mass spectra were measured in electron impact ionization mode (70 eV). Medium polarity column, BPX-50 capillary column (30 m×0.25 mm, 0.20 μm, SGE, Ringwood, Australia) was employed and head pressure of the carrier gas (helium) was 8 psig. Injection port temperature was 260 °C and the oven was programmed to raise the temperature from 80 to 260 °C at a rate of 4 °C per minute and hold at 260 °C for 5 min.

Preparation of Radical Scavenging Experiment All ginseng extracts and phenolic compounds, dissolved with phosphate-buffered saline (PBS, pH 7.4), were filtered through a filter unit of 0.45-μm pore size and diluted to standardized concentration. Black plates (Nalge-Nunc, IL, U.S.A.) were used for the prevention of possible background fluorescence and scattering in the experiment with a fluorescence probe, DHR 123.

NO-Scavenging Effect According to the method of Sreejayan and Rao,[15] 5 mM SNP in PBS (pH 7.4) was mixed with different concentrations of samples and incubated at 25 °C for 150 min in a test tube. The amount of NO produced by SNP was assayed by measuring the accumulation of nitrite, using a microplate assay method based on the Griess reaction.[16] The absorbance at 550 nm, as an index of produced nitrite, was measured after 5 min after transferring the reacted solution to 96 well plates. Curcumin was used as a NO-scavenging positive control.

O_2^--Scavenging Effect The generation and inhibition of O_2^- were measured following the method described by Ewing and Janero.[17] For the assay, the samples were pipetted into microplate wells containing 200 μl freshly-prepared 125 μM EDTA, 62 μM NBT and 98 μM β-NADH in 50 mM PBS, pH 7.4. The reaction was initiated by adding 25 μl freshly-prepared 33 μM PMS in 50 mM phosphate buffer, pH 7.4. The absorbance at 550 nm, as an index of NBT reduction, was measured after 5 min using a Tecan SPECTRAFluor (Tecan U.K., Goring-on-Thames, U.K.). L(+)-Ascorbic acid was used as a O_2^--scavenging positive control.

$ONOO^-$-Scavenging Effect $ONOO^-$-scavenging effects in the *in-vitro* system were evaluated by the method of Kooy *et al.*[18] Sample solutions and 10 μM SIN-1 were added to 5 mM DHR 123 solution, left to stand for 20 min at 37 °C and then the fluorescence of rhodamine 123, the reduced form of DHR 123, at 485 nm excitation and 535 nm emission was measured with a microplate fluorescence reader, Tecan SPECTRAFluor (Tecan U.K., Goring-on-Thames, U.K.). DL-Penicillamine was used as a $ONOO^-$-scavenging positive control.

·OH-Scavenging Effect ·OH was generated from the Fenton reaction of 50 μM $FeSO_4$ and 200 μM H_2O_2 solution[19] and the ·OH produced was detected by a fluorescence probe, DHR 123.[20] Curcumin was used as a ·OH-scavenging positive control.

RESULTS

Radical Scavenging Activities of WG, RG and SG Figure 1 shows the scavenging effects of WG, RG and SG on the NO, O_2^-, ·OH radicals and $ONOO^-$. WG had no or little effect on NO scavenging activity but RG and SG showed better inhibition effects than WG, as shown in Fig. 1A. In addition, SG exhibited better O_2^--scavenging activity than WG and RG. There was no difference in O_2^--scavenging activity between WG and RG. In particular, SG effectively scavenged $ONOO^-$ and ·OH in a concentration-dependent manner, as shown in Fig. 1C and D.

Identification of Four Phenolic Compounds For phe-

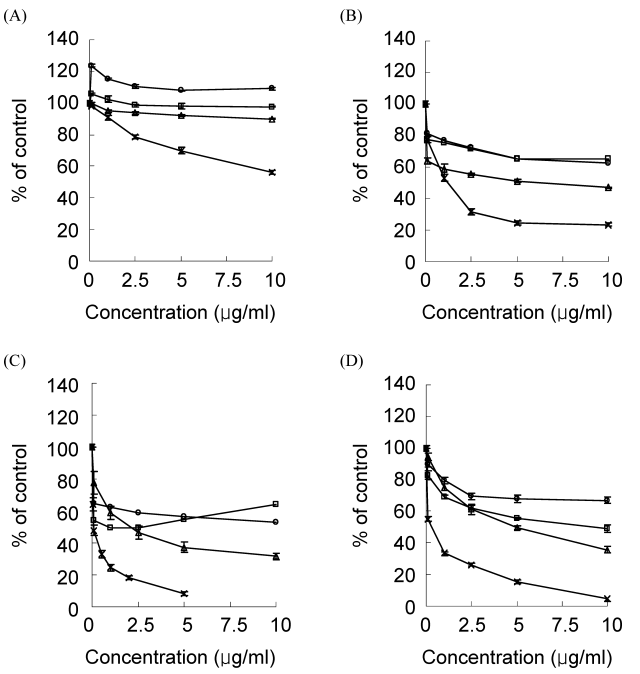

Fig. 1. Effects of Ginsengs on NO (A), O_2^- (B), $ONOO^-$ (C) and ·OH (D)
White ginseng (○), red ginseng (□), sun ginseng (△), positive control (×).

nolic compound identification, GC-MS analysis with full scan experiment in positive ionization mode was carried out and the retention order of the 4 phenolic compounds was maltol, salicylic acid, vanillic acid and *p*-coumaric acid. The mass spectra patterns of the 4 phenolic compounds were the same as those of the reference.[21] Figure 2 shows the chemical structures of the 4 phenolic compounds.

Radical Scavenging Activities of Four Phenolic Compounds Figure 3 shows the scavenging effects of maltol, salicylic acid, vanillic acid and *p*-coumaric acid on NO, O_2^-, $ONOO^-$ and ·OH. When the 5 mM SNP solution was added to each phenolic compound, NO production was reduced to 70.8 and 73.9% in vanillic and *p*-coumaric acids at the concentration of 10 μg/ml, respectively, as shown in Fig. 3A. The IC_{50} value of curcumin, the NO-scavenging positive control, was 10.5 μg/ml (Table 1). In addition, Fig. 3B shows that *p*-coumaric acid, maltol, salicylic acid and vanillic acid reduced O_2^- levels to 80.8, 73.6, 67.7 and 59.5% of the control value, respectively. The IC_{50} value of L(+)-ascorbic acid, O_2^--scavenging positive control, was 1.2 μg/ml (Table 1). Furthermore, vanillic acid, *p*-coumaric acid and maltol scavenged $ONOO^-$ strongly to 23.8, 26.3 and 26.6% of the control value, respectively (Fig. 3C). The IC_{50} value of DL-penicillamine, the $ONOO^-$-scavenging positive control, was 0.1 μg/ml (Table 1). Moreover, ·OH levels were reduced to 13.1, 13.2, 39.0 or 74.2% of the control value by treatments of maltol, *p*-coumaric acid, vanillic acid or salicylic acid, respectively, as shown in Fig. 3D. Curcumin, ·OH-scavenging positive control, showed IC_{50} value of 0.3 μg/ml (Table 1).

Contents of Phenolic Compounds in WG, RG and SG The content was calculated from the peak area ratio to that of the internal standard (*n*-eicosane). The selective ion monitoring technique was applied for the quantitative calculation of contents when mass spectra were overlapped. Linearity and repeatability of the method were tested by analyzing all samples and standards. The coefficients of correlation were >0.999 for all phenolic compounds. As shown in Table 2, the results were expressed as mg in 100 g extracts. Maltol was the major phenolic compound increased by heat treatment. The content of maltol in RG and SG was about 4 and 34 times higher, respectively, than in WG. But, esterified maltol was detected only as a trace amount. Moreover, contents of free vanillic and salicylic acids in RG were increased more than in WG, but they were decreased in SG. In addition, esterified salicylic and vanillic acids were detected as trace amounts in RG and SG. On the other hand, the free

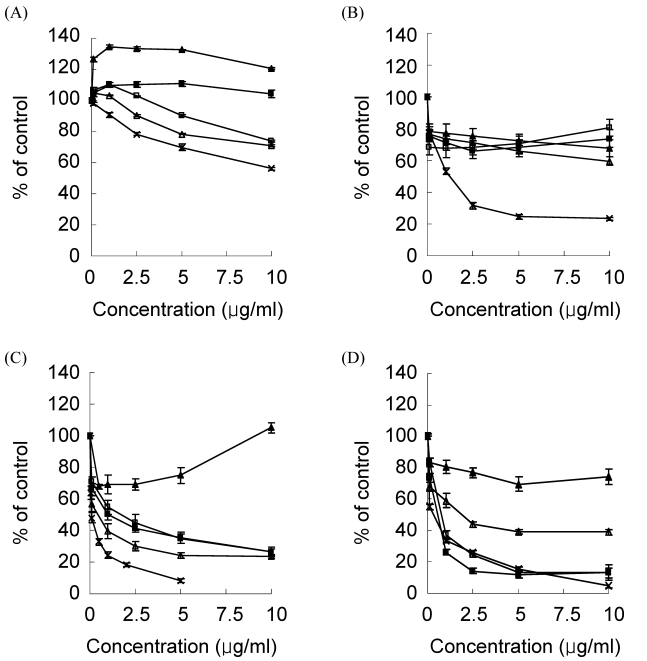

Fig. 3. Effects of Phenolic Compounds on NO (A), O_2^- (B), $ONOO^-$ (C) and ·OH (D)

Vanillic acid (△), salicylic acid (▲), *p*-coumaric acid (□), maltol (■), positive control (×).

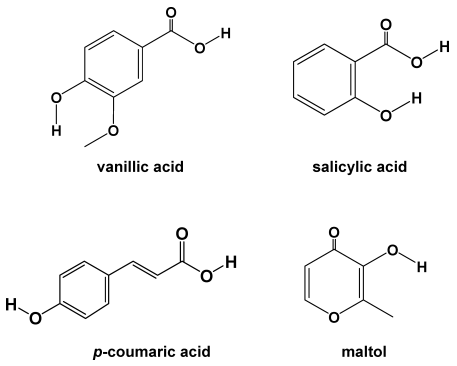

Fig. 2. Structures of Four Phenolic Compounds

Table 1. IC_{50} Values (μg/ml) against the Free Radicals

	NO	O_2^-	$ONOO^-$	·OH
White ginseng				
Red ginseng				9.3
Sun ginseng		22.2	2.1	3.4
Maltol			1.0	0.6
Salicylic acid				
Vanillic acid			0.5	1.9
p-Coumaric acid			1.7	0.8
Curcumin	10.5			0.3
L(+)-Ascorbic acid		1.2		
DL-Penicillamine			0.1	

Table 2. Contents (mg/100 g) of the 4 Phenolic Compounds in White Ginseng, Red Ginseng and Sun Ginseng

	White ginseng		Red ginseng		Sun ginseng	
	Free form	Ester form	Free form	Ester form	Free form	Ester form
Maltol	2.598		10.748	trace	94.007	trace
Salicylic acid	0.121		1.620	trace	0.394	trace
Vanillic acid	0.404	0.242	1.020	0.253	0.628	0.203
p-Coumaric acid	0.522	1.865	0.549	0.709	0.447	0.266

Trace amount (less than 0.1 mg/100 g extracts).

form of *p*-coumaric acid showed little change in content, but decreased by heat treatment in ester form.

DISCUSSION

Reactive oxygen species, generated as byproducts of biological reactions or from exogenous factors,[22] are well known to play important roles in the pathogenesis of various diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis and inflammation.[23] NO is a widespread intra- and intercellular messenger and cytotoxin.[24] The excessive production of NO participates in the pathology of inflammation, shock and injury to living tissue and produces secondary active substances by reacting with oxygen and active oxygen species. In addition, O_2^- can be directly toxic,[25] but it has a limited reactivity with most biological molecules. NO and O_2^- are known to rapidly react to form the stable $ONOO^-$. $ONOO^-$ is a powerful oxidant exhibiting a wide array of tissue damaging effects ranging from lipid peroxidation, inactivation of enzymes and ion channels *via* protein oxidation and nitration to inhibition of mitochondrial respiration.[26] Furthermore, $ONOO^-$ decomposition apparently generates $\cdot OH$, a strong oxidant.[27] $\cdot OH$ is known as an active oxygen species which reacts immediately with DNA.[28] Therefore, great effort has been made to search for safe and effective natural antioxidants which can scavenge these radicals from various plant sources.

Ginseng is one of the most widely used medicinal plants in the Orient. We classified ginseng into three types depending on how it is processed, as described above, such as WG, RG and SG. RG is known to have better pharmacological activities than WG. In addition, SG, heat-processed ginseng, has been reported to enhance the activities of WG and RG. In particular, ginseng is well known to have antioxidant activity. For example, ginseng extract has been reported to inhibit metal-induced lipid peroxidation,[29] the progression of renal failure by scavenging radicals, human low-density lipoprotein oxidation and nitric oxide synthase expression in the hippocampus of streptozotocin-induced diabetic rats.[30—32] Moreover, SG extracts also showed antioxidant activity, such as suppression of lipid peroxidation in rat brain homogenates, protection of $\cdot OH$-induced DNA strand scission and O_2^--scavenging activity.[12] Furthermore, SG exhibited stronger NO-scavenging activity than conventional WG and RG in our preliminary study. Thus, in this study, we continuously examined the NO, O_2^-, $ONOO^-$ and $\cdot OH$-scavenging activities of WG, RG and SG. As shown in Fig. 1, SG showed strong O_2^-, $ONOO^-$ and $\cdot OH$-scavenging activities compared to WG and RG. These results suppose that SG may be an effective free radical scavenger which protects against oxidative stress induced by free radicals.

Generally, ginseng saponins, known as ginsenosides and the main active component in ginseng, are believed to play a pharmacologically important role. In addition, there have been several studies about the antioxidant activity of ginsenosides. Ginsenoside-Rb_1 was found to directly interact with $\cdot OH$ and protect ischemic neurons,[33] and ginsenoside-Rd was reported to attenuate oxidative damage related to aging in senescence-accelerated mice.[34] Furthermore, ginsenoside-Rb_1 and Rc showed a tendency to increase glutathione peroxidase activity, and ginsenoside-Rc significantly decreased Cu, Zn-superoxide dismutase activity. Particularly, ginsenoside-Rh_2 remarkably increased catalase activity in the screening of antioxidative components from RG saponin.[35] On the other hand, the major ginsenosides of SG are ginsenoside-Rg_3, -Rg_5 and -Rk_1[11] and seven new ginsenosides, ginsenoside-Rk_1, -Rk_2, -Rk_3, -Rs_4, -Rs_5, -Rs_6 and -Rs_7, were isolated from SG.[36] The amounts of these ginsenosides are absent or present in trace amounts in conventional WG and RG.[11] SG, which contains these different types of ginsenosides, and its major ginsenosides have been reported to have vasorelaxation, anti-platelet aggregation, antioxidant, anti-tumor and neuroprotective activities.[12,13,37] However, in our preliminary study, almost all ginsenosides had no effect on radical scavenging activity (data not shown). Therefore, among the various pharmacological effects of ginseng, antioxidant activity, especially radical scavenging activity, can not be explained with only ginsenosides.

Phenolic compounds are commonly found in plants, and they have been reported to have multiple biological effects, including antioxidant activity. Many studies have revealed that the phenolic contents of plants can be correlated with their antioxidant activities.[38,39] Therefore, we focused on the phenolic compounds of ginseng, one of the non-saponin components, such as maltol, salicylic acid, vanillic acid, and *p*-coumaric acid. They are also known as principal antioxidant components in WG and RG. For example, they were reported to protect hepatic tissue from ethanol intoxification, but purified ginsenosides did not show these activities.[40,41] Thus, we measured the NO, O_2^-, $ONOO^-$ and $\cdot OH$-scavenging activities of phenolic compounds and their contents in WG, RG and SG. Of the 4 tested phenolic compounds, maltol showed strong $ONOO^-$ and $\cdot OH$-scavenging activities (Fig. 3).

Maltol, one of the Maillard reaction products (MRPs), was reported to be an antioxidant component of RG.[42] The Maillard reaction, the reaction between reducing sugars and amino acids or protein, is thought to be one of the major sources correlated with enhancing activity by heat treatment in various crude drugs. MRPs in ginseng were reported to be increased by steaming,[43,44] these compounds being Arg-fru-glc, Arg-fru, maltol, maltol-3-*O*-β-D-glucoside and *etc*. MRPs showed strong $\cdot OH$ inhibition and retarded the Fenton reaction.[45] In particular, there are several reports about the antioxidant activity of maltol.[46,47] Not all the MRPs in ginseng were considered in this study, but maltol could be a MRP with phenolic characteristics. Therefore, MRPs also need to be considered for an understanding of the antioxidant activity of SG. In addition, maltol was the major component to increase during processing ginseng at a higher temperature (Table 2). It seems to be produced by the reaction of free sugars or divided sugar parts from ginsenosides and glycosidic phenolic compounds, and amino acid compounds in ginseng. These findings suggest that maltol is the most abundant phenolic compound, having a strong free radical scavenging activity, in SG.

On the other hand, levels of free vanillic and salicylic acids were increased in RG compared to those in WG (Table 2). But, the contents of the compounds were not continuously increased by a higher processing temperature. Similarly, SG had more free phenolic contents than WG, but not more than RG. Thermal decomposition or reactions like dehydration in

phenolic compounds are thought to occur under high pressure and temperature, so it is thought that some phenolic contents of SG decreased more than in RG. In addition, there was a change in the levels of salicylic, vanillic and *p*-coumaric acids in WG, RG and SG, but this was not comparable to that of maltol in terms of contents (Table 2). Changes in phenolic contents were not in accordance with the reference,[14] owing to differences in extraction and sample preparation methods. Moreover, the contents of ginseng change with cultivation period, location, harvesting time and the parts utilized, *etc*.[48]

In conclusion, SG, heat processed ginseng, showed enhanced radical scavenging activities. Especially, SG showed stronger O_2^-, $ONOO^-$ and $\cdot OH$-scavenging activities than WG and RG. From the study of the quantitative analysis of radical scavenging activity tests and phenolic contents, maltol was the main radical scavenging component of SG among the 4 principal antioxidant phenolic compounds in ginseng. However, it is partly insufficient that all the radical scavenging activities explained with only maltol. Continuously, we expect to clarify the radical scavenging activity of maltol-related MRPs or new products generated during heat-processing in our future study, because these compounds in SG are not fully elucidated yet.

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