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The effect of steaming on the free amino acid contents and antioxidant activity of *Panax ginseng*

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Abstract

This study was carried out to investigate changes in the free amino acid contents and antioxidant activity of *Panax ginseng* induced by steaming at different temperatures. For this purpose, white ginseng (WG), red ginseng (RG, ginseng steamed at 100 °C) and ginseng steamed at 120 °C (SG) were prepared using an autoclave. Most free amino acids were decreased significantly by steam treatment, with the greatest reduction observed in SG. Total content of free amino acids, 17.9 mg/g in WG was reduced to 12.2 mg/g in RG and 2.79 mg/g in SG. As for Arg which is the most predominant amino acid in ginseng, the content, 10.4 mg/g in WG, decreased significantly to 1.38 mg/g in SG. In particular, β-*N*-oxalyl-*L*-α,β-diaminopropionic acid (β-ODAP), a well-known neurotoxin, was reduced by 92.9% in SG. In contrast, the level of Maillard reaction products (MRPs) increased with steam treatment, which indicates that the reduction of most amino acids is attributed to the extent of the Maillard reaction. Based on MRPs being useful antioxidants, we assayed the scavenging activity against free radicals produced by 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH). The radical scavenging activity of a ginseng extract increased with steam treatment, with the most potent activity in SG. Further, MRPs-rich fraction in SG showed powerful antioxidant activity, which indicates MRPs are major contributors to antioxidant activity enhanced by steam treatment.

Keywords: *Panax ginseng*; Amino acid; Steam treatment; β-ODAP; Maillard reaction product; Antioxidant activity

1. Introduction

Ginseng, the root of *Panax ginseng* C. A. Meyer (Araliaceae) has been widely prescribed and intensively studied as a medicinal herb. In Asia, there are two traditional preparations of ginseng, white ginseng (WG) and red ginseng (RG). WG is the dried root of ginseng, and RG is the root of ginseng which is steamed at about 100 °C and dried. RG especially shows more enhanced pharmacological activities than WG (Kim et al., 2000; Nam, 2005). The differences in biological activities of WG and RG may result from a change of the chemical constituents that occurs during steam treatment. It is reported that unique ginsenosides in RG are ginsenosides Rg_3, Rg_5, Rg_6, Rh_2, Rh_3, Rh_4, Rs_3 and F_4 which are less polar (Kim et al., 1996; Kim et al., 2000).

Our research group has reported a new processed steamed ginseng (SG), produced by steaming WG at high temperature (120 °C). We have found that SG possesses better biological activities than RG in free radical scavenging, endothelium-dependent relaxation, anti-inflammatory and anti-tumor effects (Keum et al., 2000; Kim et al., 2000; Kwon et al., 2001). In terms of chemical constituents, the amount of nonpolar ginsenosides such as Rg_3, Rg_5 and Rk_1 is hundreds of times greater than those in RG (Kwon et al., 2001). The nonpolar ginsenosides are produced by deglycosylation and/or dehydration when ginseng is steamed (Ando, Tanaka, & Shibata, 1971; Kim et al., 1996). However, so far our studies have mainly focused

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on ginsenosides, and other constituents of SG have been studied in less detail.

Recently, dietary non-protein amino acids were implicated as potential factors in human diseases of unknown etiology. In particular, β-*N*-oxalyl-*L*-α,β-diaminopropionic acid (*β*-ODAP), a naturally occurring non-protein amino acid, was suggested to cause neurolathyrism, a primary upper motor neuron disease, after over-consumption of grass pea seeds (*Lathyrus sativus* L.) during drought triggered famine episodes in Ethiopia, India and Bangladesh (Getahun, Mekonnen, TekleHaimanot, & Lambein, 1999). In addition to its neurotoxicity, *β*-ODAP was reported to be gliotoxic, causing the lysis of glial cells in neonatal rat astrocytes (Bridges, Hatalski, Shim, & Nunn, 1991). β-ODAP is found in leguminous plants, and ginseng is the only non-legume plant in which it is known to be present (Kuo, Ikegami, & Lambein, 2003). β-ODAP has been detected in ginseng roots of various species of different origins including *P. ginseng*, *P. notoginseng* and *P. quinquefolius* (Long, Ye, & Xing, 1996).

On the other hand, it has been reported that during thermal treatment of raw ginseng below 100 °C, browning compounds, i.e., Maillard reaction products (MRPs) are formed from the reaction of the carbonyl group of a reducing sugar with the free amino group of amino acids (Li, Zheng, Liu, & Zhang, 1999; Suzuki et al., 2004). MRPs are known to be useful as antioxidants in foods and herbal drugs. In particular, Samaras et al. found that antioxidant activity increased with the intensity of heating, in parallel with color formation and MRPs were responsible for the majority of the antioxidant activity, in highly roasted malts (Samaras, Camburn, Chandra, & Gordon, 2005). Therefore, it is worth measuring the changes of the MRP level and antioxidant activity induced by steaming ginseng and investigating the contribution of MRPs to antioxidant activity in steamed ginsengs.

The objectives of this study were to determine the changes in the free amino acid contents including *β*-ODAP, the MRP level and the antioxidant activity induced by steaming process in order to establish the effect of steaming thereon.

2. Materials and methods

2.1. Chemicals

Phenyl isothiocyanate (PITC), L-allylglycine, allophycocyanin, trolox and standard amino acids except β-ODAP were purchased from Sigma (St. Louis, MO, USA). Triethylamine was from Aldrich (St. Louis, MO, USA). 2,2′-Azobis-(2-amidinopropane) dihydrochloride (AAPH) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). β-ODAP was a generous gift from Dr. Zhi-Xiao Li at Lanzhou University, China.

2.2. Plant materials and preparation of steamed ginseng

The dried rootlet of *P. ginseng*, i.e., WG, was cultured in Keumsan, South Korea. The steamed ginsengs, i.e., RG and SG, were prepared by steaming WG at 100 °C and 120 °C for 3 h by using an autoclave, respectively, followed by drying. The WG, RG and SG were pulverized with a Shinil (Hwasung, Korea) model SFM-555SP electric mill.

2.3. Sample preparation for HPLC analysis

Two hundred milligrams of each sample was extracted by sonification in 10 mL of 70% EtOH for 1 h, using a Branson (Danbury, CT, USA) model Bransonic 2210 sonicator. Fifty microlites of internal standard (100 μmol/ml L-allylglycine) was added. The extracts were centrifuged at 6000*g* for 20 min. The supernatants were pooled and evaporated under reduced pressure to dryness. To this, 1 ml of deionized water was added, respectively.

2.4. PITC derivatization

One hundred microlitre aliquots of each ginseng extract were derivatized with PITC and dissolved in 500 μl buffer A (0.1 M NH_4OAc, pH 6.5) for HPLC analysis (Khan, Kuo, Kebede, & Lambein, 1994).

2.5. HPLC Instrumentation and analysis

The HPLC system consisted of two Hitachi (Tokyo, Japan) model L-7100 pumps coupled with a Rheodyne (Cotati, CA, USA) model 7125 injector, a Hitachi photodiode array detector model L-7450 A, a Hitachi column oven model L-7300 and a Phenomenex (Torrance, CA, USA) Degassex™ Model DG-4400 degasser. Separations were carried out on an Alltech (Deerfield, IL, USA) Apollo C18 column (250× 4.6 mm i.d., 5 μm) and a Phenomenex Synergi Hydro-RP column (150× 4.6 mm i.d., 4 μm). The column temperature was 43 °C. A gradient elution system of buffer A (0.1 M NH_4OAc, pH 6.5) and buffer B (0.1 M NH_4OAc-ACN-MeOH = 44:46:10; v/v/v, pH 6.5) was used [0% buffer B (0 min); 10% buffer B (15 min); 40% buffer B (30 min); 50% buffer B (40 min); 100% buffer B (50 min)] (Khan et al., 1994). The flow rate was 1 ml/min.

2.6. Sample preparation for MRP level measurement and allophycocyanin assay

Ten grams of each sample were extracted by sonification with 70% EtOH for 1 h. The solvent was then evaporated *in vacuo* to give each extract of WG, RG and SG, each with a yield of about 30%. SG extract (3 g) was suspended in water and the water-soluble polysaccharide fraction was separated by Mitsubishi Chemical (Tokyo, Japan) Diaion HP 20 column chromatography using water as the eluent followed by elution with MeOH. Each fraction was evaporated *in vacuo* to give the water eluate (SG-DW) (2.3 g) and the MeOH eluate (SG-MeOH) (260 mg). SG-MeOH was suspended in water and successively partitioned with diethyl ether and *n*-BuOH. Each fraction was dried under reduced pressure and the ether fraction (SG-MeOH-Ether)

(65 mg), *n*-BuOH fraction (SG-MeOH-BuOH) (160 mg) and the residue (SG-MeOH-Residue) (9.5 mg) were obtained.

2.7. Measurement of MRP levels

The extent of browning, or MRPs generation, was measured as previously reported with slight modification (Samaras et al., 2005). Three ginseng extracts were dissolved in 50% EtOH (1 mg/ml), and the absorbance at 420 nm was measured using a Shimadzu (Kyoto, Japan) UV-1200 UV–vis spectrophotometer with Shimadzu quartz cuvettes of optical length 10 mm.

2.8. Allophycocyanin assay

According to the method of Courderot-Masuyer et al., the reaction mixture containing 37.5 nM allophycocyanin, 3 mM AAPH, and aqueous solution of test sample in 75 mM PBS (pH 7.0) was incubated at 37 °C (Courderot-Masuyer, Dalloz, Maupoil, & Rochette, 1999). The concentration of each sample was 10 μg/ml and 200 μl of the reaction mixture was added to each well of Nunc (Roskilde, Denmark) model F96 Microwell™ plates (surface: Maxisorp, color: Black). The fluorescence obtained just before the addition of the radical generator AAPH was used as the 100% value for that sample. Loss of fluorescence was measured every 10 min at an emission wavelength of 635 nm and an excitation wavelength of 590 nm using Tecan (Goring-on-Thames, UK) SPECTRAFluor spectrometer.

2.9. Statistical Analysis

The mean and standard deviation were calculated for all experiments. The data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test to determine whether the mean of the respective amino acid content in WG, RG and SG was significantly different from one another. In all cases, a P value of <0.001 was used to determine the significance. For taurine, a P value of <0.05 was applied.

3. Results and discussion

3.1. Analysis of free amino acids in WG, RG and SG

Fig. 1 shows typical chromatograms of WG, RG and SG. In Figs. 1(A–C), (analyzed on Apollo C18 column), Thr and GABA peaks (peak nos. 11 and 12) were not separated. These two peaks were separated on Synergi Hydro-RP column, as shown in Fig. 1(A-1). Table 1 summarizes the contents of 21 free amino acids in WG, RG and SG, and the results of Duncan's test for means comparison are also included. Total content of free amino acids, 17.9 mg/g in WG was reduced to 12.2 mg/g in RG and 2.79 mg/g in SG. The content of all the amino acids except for Asp and taurine, decreased with the intensity of the steam treatment. Exceptionally, Asp increased when steamed, and taurine showed no statistically significant changes (even $P < 0.05$) induced by steam treatment. All the amino acids have significantly different ($P < 0.001$) mean values in WG, RG and SG, except for the amino acids Asp, Glu, Gly, Gln, taurine and Ala. As for Asp, Glu, Gly, Gln and Ala contents, each mean value of SG showed significant differences ($P < 0.001$) with that of WG but not that of RG. The predominant components in free amino acids, β-ODAP (1.77 mg/g), Asn (1.25 mg/g), Gln (0.708 mg/g), Arg (10.4 mg/g) and GABA (0.876 mg/g), decreased significantly to β-ODAP (0.126 mg/g), Asn (0.059 mg/g), Gln (0.008 mg/g), Arg (1.38 mg/g) and GABA (0.161 mg/g) after steaming at 120 °C.

In case of β-ODAP, a great reduction in the content was observed in SG, compared to that in RG (1.19 mg/g). In other words, β-ODAP was reduced by only 32.8% after steaming at 100 °C, but abruptly reduced by 92.9% after steaming at 120 °C. This result agrees well with the report that the β-ODAP in dry seed of grass pea (*Lathyrus sativus*) was significantly reduced ($P < 0.05$) by 39% after autoclaving for 30 min (Akalu, Johansson, & Nair, 1997). The reduction in β-ODAP content induced by steaming at high temperature is due to its instability in heat (Akalu et al., 1997). The remarkable decrease of β-ODAP in ginseng by steaming at 120 °C is very noteworthy considering the neurotoxic properties of β-ODAP. Furthermore, taurine, which is known to have a neuroprotective effect, was not significantly influenced by steam treatment.

3.2. Analysis of MRP level and antioxidant activity in WG, RG and SG

MRP levels for WG, RG and SG were determined by the absorbance at 420 nm, as shown Fig. 2(a). A wavelength of 420 nm is representative of the wavelengths used to monitor colored compounds formed by the Maillard reaction. The MRP levels in WG, RG and SG were 0.03, 0.20 and 0.44, respectively. The MRP level in SG was 14.7 times higher than that in WG and 2.2 times higher than that in RG. This increase of MRP level with the intensity of steaming is likely to be related to the degree of the Maillard reaction (Suzuki et al., 2004). Further, the decrease of amino acid contents induced by steaming, as shown before, is attributed to the extent of the Maillard reaction. In other words, the sharp decrease of amino acid contents and remarkable increase of MRP level in SG, compared to those in RG, were considered to result from accelerated Maillard reaction, which is in line with the report that Maillard reaction was accelerated with the intensity of heating (Turkmen, Sari, Poyrazoglu, & Velioglu, 2006).

MRP levels for sub-fractions of SG were SG-DW 0.09; SG-MeOH 0.46; SG-MeOH-Ether 0.45 [a phenolic compound-rich fraction] (Jung, Jeon, & Bock, 2002); SG-MeOH-BuOH 0.32 [a saponin-rich fraction] (Kim et al., 2000); and SG-MeOH-Residue 1.00, as shown in

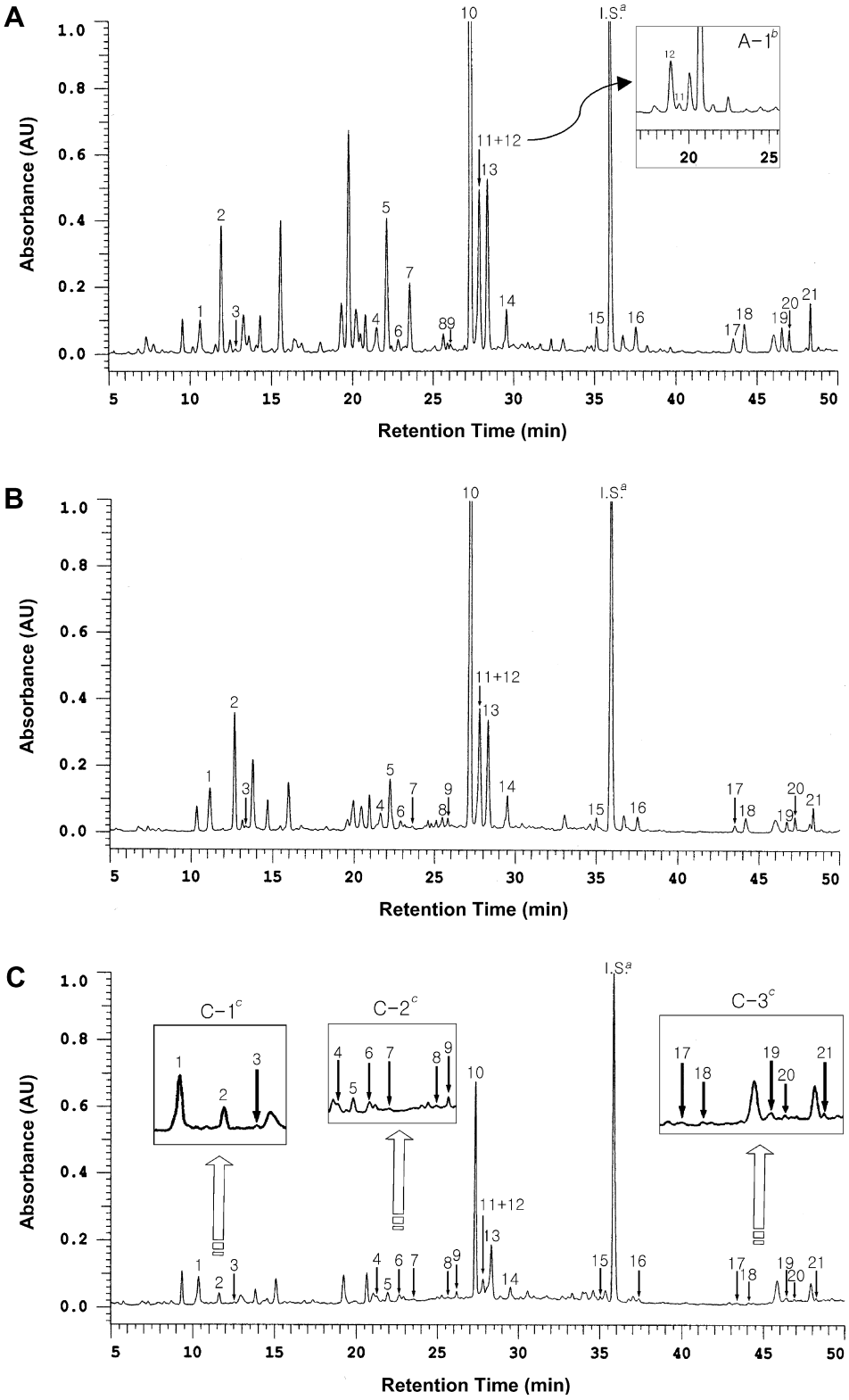

Fig. 1. Chromatograms of WG (A), RG (B) and SG (C): analysis on Apollo column (Peak identity: see Table 1). (a) I.S. designates an internal standard peak. (b) Figure A-1 shows clear separation of Thr and GABA on Synergi Hydro RP column. (c) Figure C-1, C-2 and C-3 are ones magnified in order to clearly show small peaks in Figure C.

Fig. 2(b). The MRP level in SG-MeOH was 5.1 times higher than that in SG-DW, and the MRP level in SG-MeOH-Residue was 2.2 times higher than that in SG-MeOH-Ether and 3.1 times higher than that in SG-MeOH-BuOH. This result indicates that SG-MeOH-Residue is a MRPs-rich fraction. Since the content of

Table 1
Mean values and standard deviations (SD) of neuroactive amino acid β-ODAP and other free amino acids (mg/g of dry matter) in WG, RG and SG[a]

Peak no.	Amino acid	WG		RG			SG		
		Mean	SD	Mean	SD	Relative content[d] RG/WG	Mean	SD	Relative content[d] (SG/WG)
1	Asp	0.341 B	0.003	0.402 A	0.005	1.18	0.399 A	0.021	1.17
2	β-ODAP	1.77 A	0.034	1.19 B	0.028	0.671	0.126 C	0.006	0.071
3	Glu	0.017 A	0.046	0.013 B	0.001	0.319	0.010 B	0.001	0.247
4	Ser	0.174 A	0.003	0.103 B	0.003	0.591	0.052 C	0.004	0.299
5	Asn	1.25 A	0.023	0.413 B	0.017	0.330	0.059 C	0.004	0.047
6	Gly	0.032 A	0.001	0.012 B	0.002	0.389	0.012 B	<0.001	0.380
7	Gln	0.708 A	0.008	0.007 B	0.001	0.010	0.008 B	0.002	0.012
8	His	0.163 A	0.005	0.048 B	0.001	0.295	0.011 C	<0.001	0.070
9	Taurine[b]	0.057 A	0.001	0.056 A	0.010	0.982	0.059 A	0.001	1.035
10	Arg	10.4 A	0.268	8.17 B	0.830	0.784	1.38 C	0.173	0.132
11	Thr	0.147 A	0.002	0.100 B	0.005	0.680	0.017 C	0.004	0.119
12	GABA[c]	0.876 A	0.017	0.659 B	0.038	0.753	0.161 C	0.010	0.184
13	Ala	0.580 A	0.021	0.436 B	0.023	0.751	0.393 B	0.006	0.678
14	Pro	0.171 A	0.001	0.141 B	0.004	0.823	0.042 C	0.002	0.244
15	Tyr	0.175 A	0.004	0.081 B	0.002	0.461	0.011 C	0.001	0.066
16	Val	0.162 A	0.001	0.074 B	0.001	0.458	0.028 C	0.003	0.173
17	Ile	0.095 A	0.003	0.043 B	0.005	0.449	0.009 C	0.003	0.094
18	Leu	0.257 A	0.003	0.107 B	0.003	0.415	0.005 C	0.001	0.019
19	Phe	0.168 A	0.004	0.058 B	0.003	0.342	0.005 C	0.001	0.031
20	Trp	0.130 A	0.001	0.061 B	0.005	0.468	0.002 C	<0.001	0.013
21	Lys	0.132 A	0.002	0.052 B	0.001	0.394	0.002 C	0.001	0.014
Total content		17.9		12.2			2.79		

[a] n = 4; Means in the same row with different letters are significantly different ($P < 0.001$).
[b] There is no significant difference ($P > 0.05$) between WG, RG and SG.
[c] GABA: γ-aminobutyric acid.
[d] Relative content = [the content of an amino acid in RG or SG]/[the content of an amino acid in WG].

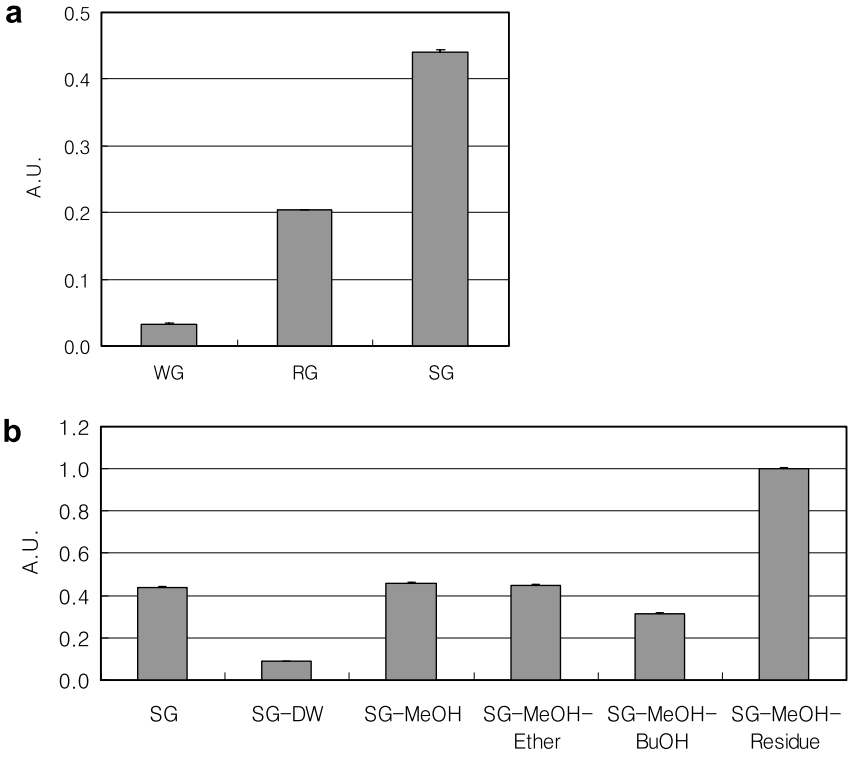

Fig. 2. Absorbance at 420 nm of extracts: (a) WG, RG and SG, (b) Sub-fractions of SG.

SG-MeOH-Residue is relatively low in SG, as shown in Section 2.6., SG shows relatively low MRP level in comparison with high MRP level of SG-MeOH-Residue.

In addition, we performed allophycocyanin assays for WG, RG and SG. In this assay, we measured the fluorescence intensity of allophycocyanin, a protein with natural

fluorescence. AAPH produces free radicals at a constant and measurable rate of its temperature-dependent decomposition and these free radicals are known to react with oxygen to yield peroxyl radicals (Wayner, Burton, Ingold, Barclay, & Locke, 1987). Following treatment with AAPH, the intrinsic fluorescence of allophycocyanin was rapidly diminished, reflecting the oxidation of allophycocyanin (Nakagawa, Yokozawa, Terasawa, Shu, & Juneja, 2002). Fig. 3(a) shows the decrease in allophycocyanin fluorescence by AAPH-induced peroxyl radicals. In the absence of sample (control), the intrinsic allophycocyanin fluorescence was rapidly diminished to 0% after 50 min. When ginseng samples were present in the reaction mixture, a right shift in the curve was noted, indicating AAPH-induced peroxyl radical scavenging activities of these ginseng extracts. The fluorescence values for WG, RG and SG were about 35, 62 and 78% at 60 min and that of trolox, a positive control, was about 66% at 60 min. This result indicates that, SG retarded the fluorescence diminishment much more than WG and RG, and that the free radical scavenging activity of ginseng increased with the intensity of steam treatment.

This assay was also performed with SG-MeOH sub-fractions, i.e., SG-MeOH-Ether, SG-MeOH-BuOH and SG-MeOH-Residue. As shown in Fig. 3(b), SG-MeOH-Residue (MRPs-rich fraction) was the most potent against AAPH-induced protein damage. This suggests that MRPs may be major contributors to the enhanced antioxidant activities against AAPH-induced free radicals.

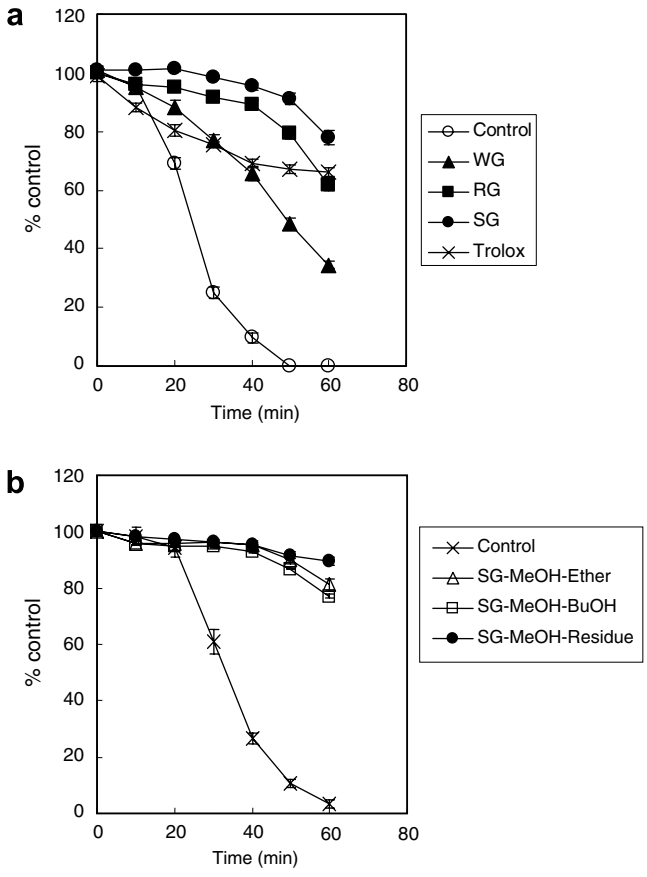

Fig. 3. Time-response curve of ginseng extracts on allophycocyanin quenching induced by AAPH: (a) WG, RG and SG; (b) Sub-fractions of SG.

4. Conclusions

Most free amino acids in ginseng decreased with the intensity of steaming and the contents in SG were sharply diminished, compared with those in RG. In particular, β-ODAP was reduced by only 32.8% in RG, whereas abruptly reduced by 92.9% in SG, which results from its instability to heat. The reduction in most free amino acids, except for β-ODAP, was attributed to the acceleration of Maillard reaction. SG had the highest MRP level and the most potent antioxidant activity. By assaying the sub-fractions of SG, we found that MRPs in SG may be responsible for the majority of the antioxidant activity.

Therefore, high quality of ginseng can be obtained by steaming ginseng at higher temperature (120 °C), in that a neurotoxin β-ODAP sharply decreases and the antioxidant activity remarkably increases.

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