STUDIES ON THE ANTI-OXIDANT COMPONENTS OF KOREAN GINSENG

Byung Hoon Han, Myung Hwan Park, Lin Keun Woo, Won Sick Woo and Yong Nam Han*

Natural Products Research Institute, Seoul National University

**Korea Ginseng Research Institute*

Seoul, KOREA

Abstract

This paper is concerned with the studies on the effective components of anti-oxidant activity, with a view to demonstrate the anti-aging activity of Korean ginseng. Feeding the extract of Korean ginseng or its effective component to mice inhibited strongly the induction of lipid peroxidation produced by ethanol intoxication. From the extract of Korean red ginseng, one effective component Compound A, mp.143, $C_6H_6O_3$ was isolated by chromatographic purification and its chemical structure was determined as 2-methyl-3-hydroxy-γ-pyrone(maltol).

Introduction

Lipid peroxide in cells and biological anti-oxidants are frequently discussed in connection with the various diseases of aging such as liver disease[1-2], diabetes melitus[3], arterosclerosis[4-6], ophthalmic disease[7], and nerve aging[8].

Recently, the biological anti-oxidants which reduce the lipid peroxidation in cells are being investigated by gerontologists, since the lipid peroxide in cells are known as the precursor of the lipofuscin pigment[8-12]. This pigment is known lately to be increased in parallel with the cellular aging[3,8,11]. On the other hand, Korean ginseng has already been known to the Oriental peoples as being the elixir of life and as the medicine of long life from several thousand years ago. Speculation of those ethnobotanical efficacy suggests fully the anti-aging activity for the efficacy of Korean ginseng. However, no article has ever concerned with the subject. Present paper deals with the demonstration of anti-oxidant activity from the extracts of Korean red ginseng and white ginseng in a view to prove anti-aging activity and the characterization of one effective component, Compound A, mp. 143, $C_6H_6O_3$, isolated from the extract of red ginseng, as being 2-methyl-3-hydroxy-γ-pyrone(maltol).

Materials and Methods

Materials: Thiobarbituric acid (TBA) was synthetized from diethyl-malonate and thiourea in our laboratory. Diphenyl-picryl-hydrazyl(DPPH), a stable free radical reagent was also synthetized by the method of S. Goldschmidt[13]. Sodium dodecyl sulfate was recrystallized from ethanol before use.

Animals: Male mice weighing 25-30gm were used without considering their strains and maintained by normal diet purchased from market. One experimental group consisted of 5-7 animals and the total body weight of each experimen-

tal group was equalized as possible.

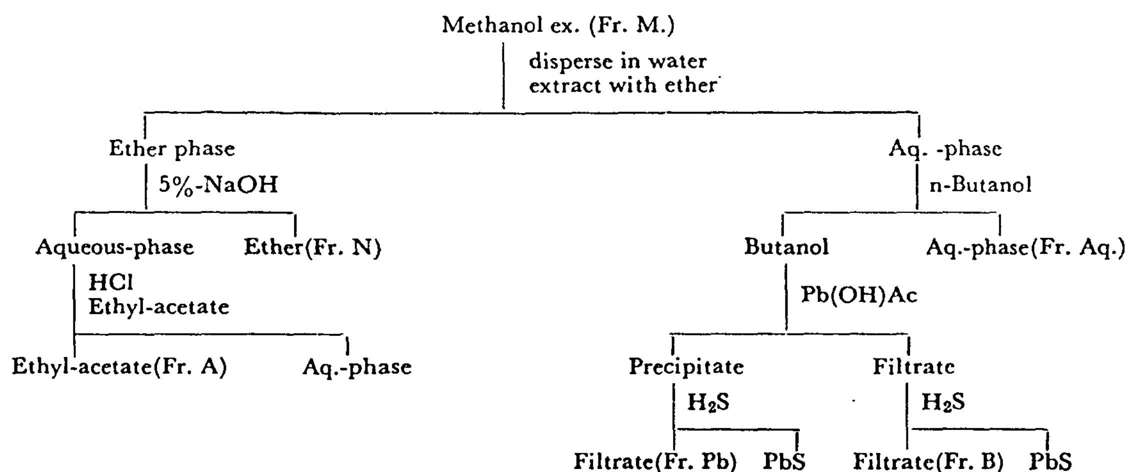
Medication: Mice were received the ginseng samples as saline solutions or suspensions once daily for three days. During the medication the animals were fed with normal diet. Control mice were fed with the equal amount of saline.

Acute ethanol intoxication (Induction of lipid peroxidation): The lipid peroxidation was induced by the method of G.H. Kalish[14]. The animals which had been received the medication for three days were fasted for eight hours after the last medication and then received a single dose of 0.3ml 50%-ethanol per 20gm mouse. The animals were fed freely with normal diet and water 30 minutes after the ethanol intoxication. Control mice were fed with saline instead of ethanol. Twenty-four hours after the ethanol treatment the animals were decapitated, blood in liver was removed as possible by bleeding and the livers were removed in order to assay the lipid peroxide content by thiobarbituric acid method (TBA-method).

Assay of lipid peroxide content in liver (TBA-value): The lipid peroxide content in liver was assayed by F. Masugi procedure[15] of thiobarbituric acid method after some modification as follows. The livers in one group of animals were pooled, washed with saline, weighed and homogenated for three minutes in an ice cooled motor driven homogenizer after addition of five volume of M/20–phosphate buffer(pH 7.4). To a 0.5ml homogenate in a glass stoppered tube, 0.4ml of 10%–sodium dodecyl sulfate solution was added and incubated at room temperature for 30 minutes. To the incubated mixture a 2ml of 0.1N-HCl and 1.0ml of 1%–thiobarbituric acid solution were added and heated for 50 minutes in a 95°C water bath to develop the red color of TBA-pigment. After cooling the TBA-pigment was extracted with 5ml butanol. The butanol layer was obtained by centrifugation at 3000 rpm for ten minutes and its optical density was measured at 532nm. The lipid peroxide content in liver is expressed as TBA-value (A_{532}/gm.wet weight of liver).

Assay of free radical quenching activities[16]: Diphenyl-picryl-hydrazyl, a stable free radical reagent (DPPH) was synthetized in our laboratory by the method of S.Goldschmidt[13]. DPPH was dissolved in ethanol and diluted appropriately until it showed absorbancy A_{517} 0.80. To a 3ml DPPH solution 0.1ml of ginseng extract or the solution of ginseng component was mixed and the absorbancy decrease during ten minutes at 517nm was measured as the free radical quenching activity.

Fractionation of ginseng extract: 120gm of fresh ginseng root or red ginseng were extracted by boiling with methanol and concentrated in vacuo to give a syruppy extract. The extract was fractionated by solvent partitioning and by lead acetate precipitation process as shown in Scheme-1 and each fraction was tested for its free radical quenching and anti-oxidant activity. The final



Scheme-1 : Fractionation of ginseng extract.

volumes of every fraction were adjusted to 30ml by dissolving or suspending in saline.

Isolation of compound A(maltol) from red ginseng: One kg of Korean red ginseng extract which was supplied from the Korea Ginseng Research Institute was dispersed in a small volume of water and partitioned with ether. The ether soluble fraction was extracted with 5% NaOH solution. The alkaline extract was acidified by d-HCl and extracted with ethyl acetate. The ethylacetate phase washed with water, dried over anhydrous sodium sulfate and concentrated to give 32gm ether soluble acidic fraction(Fr. A). The Fr. A(8gm) was chromatographed on a silica gel column (250gm) using benzene: acetone (4:1)as eluent. A main component giving a red violet spot on TLC by $FeCl_3$ was isolated in a pure state, recrystallized twice from acetone to give fine needles, mp. 143°C,$C_6H_6O_3$ and designated it as Compound A. It gives a positive iodoform test, reacts with diazomethane, sublimes completely when it is heated slowly above 120°C and gives a red violet with $FeCl_3$. UV-absorption maximum is 277nm(E; 4300) and is shifted to 322nm(E;3800) by the addition of alkali solution. Mass spectrum shows molecular ion at m/e 126. PMR gives six protons $\delta^{TMS}_{CDCl_3}$; 2.36(3H,*s*) of methyl group, 6.41(1H,*d*, J = 6Hz) and 7.68(1H,*d*, J = 6Hz) of olefinic AX protons and 7.0(1H,*br.*) of hydroxyl proton. CMR in pyridine gives six carbon peaks at 14.2, 113.3, 143.3, 149.5, 154.1 and 173.2ppm (TMS) which are superimposable with the spectrum of maltol. IR spectrum gives several strong absorptions at V_{OH} 3270, V_{CH} 3070, $V_{C=O}$ 1660, $V_{C=C}$ 1570 which is also superimposable with the spectrum of standard maltol. Analysis found; C,57.1%, H, 4.87%, $C_6H_6O_3$ requires C, 57.1%, H, 4.76%. This compound shows a strong inhibitory activity on ethanol induced lipid peroxidation in mice livers and shows strong free radical quenching activity (DPPH reduction).

Acetylation of compound A(maltol acetate): The compound A(380mg) was dissolved in a mixture of 5ml pyridine and 5ml acetic anhydride and kept at room temperature for 24 hours. Excessive reagent was removed by vacuum distillation to obtain crystalline residue in a quantitative yield. Recrystallization from ether gave fine needles, $C_8H_8O_4$, mp.55°C which gives no reaction with $FeCl_3$. PMR of acetate gives eight protons $\delta^{TMS}_{CDCl_3}$; 2.30(3H,*s*) of methyl group, 2.10(3H,*s*) of acetyl group and 6.41(1H,*d*,J = 6Hz) and 7.68(1H,*d*,J = 6Hz) of olefinic AX protons.

IR-spectrum of acetate gives strong peaks due to acetyl group at $V_{C=O}$ 1750 and δ_{CH} 1250. Analysis found; C, 57.1%, H, 4.9%. $C_8H_8O_4$ requires; C, 57.1%, H, 4.79%.

Results and Discussions

1) In our preliminary experiment, it was confirmed that the methanolic extracts of Korean ginseng(both red and white ginseng) showed anti-oxidant activity when it was checked by the inhibition of ethanol induced lipid peroxidation in mouse liver. The extract showed also free radical quenching activity. The anti-oxidant activity of the ginseng extract was considered to be associated with the free radical quenching activity of the extracts, since both activities were abolished by diazomethane treatment on the extracts(Table1). Therefore both activity parameters were adopted for tracing the active principles in the purification process, choosing one of them in proper convenience.

2) The dammarane triterpene glycosides of Korean ginseng have been the focus of ginseng sciences. Therefore we examined the anti-oxidant activity and free radical quenching activity of the purified dammarane glycosides such as ginsenoside Rb_1, Rc, Re, and Rg_1. However, they were all found to be negative in the both tests.

3) The methanol extract of red ginseng and fresh ginseng showed UV-absorption maximum at 278nm which gave bathochromic shift to 320nm by the addition of alkali. This shift, however, disappeared upon the same treatment when the extract was pretreated with diazomethane. The diazomethane treatment on the extract blocked also the anti-oxidant activity, suggesting the phenolic nature as the entity of the anti-oxidant prin-

ciples.

4) The ginseng extracts were fractionated by the procedures shown in Scheme-I. The final volumes of each fraction were equalized in order to compare directly with their anti-oxidant activity. The free radical quenching activities and the anti-oxidant activities of each fraction are summarized in Table 1 and 2.

Table 1. Free radical quenching activities of ginseng fractions; To a 3ml diphenyl-picryl-hydrazyl solution in a dilution of A_{517} 0.80 mixed 0.1ml of each fractions of fresh ginseng extract and the decrease in absorption during ten minutes were recorded. Experiment with the red ginseng fractions gave result of same tendency.

Fractions	Decrease of A_{517} during ten minutes
MeOH-ex(Fr. M)	—*
n-Butanol phase	0.71
Fr. Pb	0.24
Fr. B	0.01
Ether phase	0.72
Fr. N	0.08
Fr. A	0.43
Fr. Aq	—*
Ginsenoside Rb_1, Rc, Re and Rg_1	0.00
Fr. M and diazomethane	0.00

—* indicate the formation of precipitate during reaction with DPPH-reagent.

As shown in Table 1, the major portion of free radical quenching activities of fresh ginseng distributed in the ether soluble acidic fraction(Fr.A) and in the glycosidic fractions(Fr. Pb) which forms precipitate with basic lead acetate solution. A similar tendency in the distribution of the activities were observed in the fractions of red ginseng extract. Table 2 show the inhibitory effect of each fraction of fresh ginseng extract on the ethanol induced lipid peroxidation in the mouse liver. The activity distribution in the fractions shows similar tendency with that of free radical quenching activities in Table 1,except Fr. B due to unaccountable reason. Both activities were abolished by diazomethane pretreatment.

5) The ether soluble acidic fraction(Fr.A) of red ginseng gave a clearly separated spot on

Table 2. The inhibitory effect of fresh ginseng fractions on the ethanol induced lipid peroxidation of mouse liver is recorded as the TBA-value per gm of liver wet weight. The methanol extract of fresh ginseng 120gm was fractioned as Scheme-I. The final volume of each fractions were all adjusted to 30ml and 0.1ml of the solutions per 10gm body weight of mouse were administered orally for three days.

Fractions	TBA-values
Normal	9.1
Ethanol control	39.8
Fr. M	22.0
Fr. B	17.0
Fr. Pb	16.5
Fr. N	42.0
Fr. A	15
Fr. Aq.	38
Fr. M and diazomethane Ginsenoside Rb_1, Rc, Re and Rg_1	—*

—* indicate a separate experiment in which the inhibitory activity of those pure ginsenoside samples on the ethanol induced lipid peroxidation in the mouse liver were shown to be negative.

TLC with benzene : acetone(4 : 1) system which gives colour reaction with $FeCl_3$ and reduces DPPH free radical. The Fr. A chromatographed over silica-gel column to give ferric chloride positive substance in a crystalline state mp.143°C and designated it as Compound A. The compound A is detected only in the extract of red ginseng. This substance showed free radical quenching activity and a strong inhibitory activity on the ethanol induced lipid peroxidation as shown in Tables 3 and 4.

Table 3. The free radical quenching activity of Compound A; To 3.0ml of ethanolic solution of diphenyl-picryl-hydrazyl free radical reagent (A_{517}= 0.80) was added 0.1 ml of compound A in ethanol. The decrease of DPPH absorption at 517nm during ten minutes was measured.

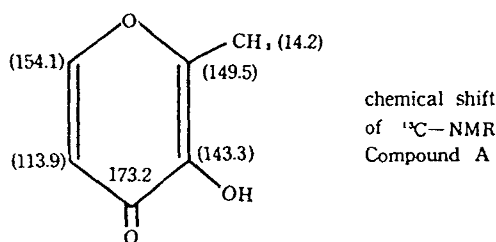
Concentration of compound A	Decrease in absorption at 517nm
1.58×10^{-6}M	0.050
4.74×10^{-6}M	0.107
9.48×10^{-6}M	0.175

Table 4. Anti-oxidant activity of compound A. Compound A or tocopherol acetate was administered orally to mice once daily for 3 days. Eight hours after the last medication 50%-ethanol was administered orally and the lipid peroxide content in the mouse liver was assayed by thiobarbituric acid method, 24 hours after the ethanol intoxication.

Experiment No.	Normal	Ethanol control	Compound A			Tocopherol acetate	
			0.01mg	0.1mg	1.0mg	0.2mg	4mg
1	10.1	38.52		9.6		11.16	9.36
2	8.95	31.4		11.04			
3	9.67	38.76		18.36			
4	10.44	32.4		12.5			
5	9.67	38.76	24.72	18.36	17.40		

As shown in table 3 and 4, compound A shows a strong free radical quenching and anti-oxidant activity. The oral administration of compound A in the doses of 0.1mg per mouse inhibited the ethanol induced elevation of TBA-value to almost control level. Concurrent experiment with tocopherol acetate showed almost equivalent potency. The activities show also dose dependencies.

6) Compound A is negative in nitrogen test, showing a phenolic characteristic in its UV-spetrum. Considering various spectral data of compound A and its acetate two kinds of γ-pyrone derivatives such as 2-methyl-3-hydroxy-γ-pyrone or 2-hydroxy-3-methyl-γ-pyrone were tentatively suggested for the probable structure of compound A.



chemical shift of ^{13}C−NMR Compound A

The compound A shows a strong chelating activity with ferric chloride giving an intense colour reaction (red violet). This suggests the vicinal location of the carbonyl group and hydroxyl group in compound A. Hence direct comparison with the authentic specimen of 2-methyl-3-hydroxy-γ-pyrone gave superimposable spectra of IR and ^{13}C-NMR.

7) Although the isolation of active principle from fresh ginseng has been unsuccessful until now, it could be considered that fresh ginseng should contain some other series of active principles which could be transformed into compound A during heat treatment. Our further work is now progressing to isolate these active principles.

Acknowledgement

The author is grateful to Professor Osamu Tanaka for his kind measurement of ^{13}C-NMR spectrum.

References

1. Recknagel, R. O., Ghoshal, A. K.: Lab. Invest., **15**, 132(1966).
2. Masugi, F. and Nakamura, T.: Internatl. J. Vit. Nutr. Res. **46**, 187(1976).
3. 坂本信夫：過酸化脂質の問題点, p.43(東京田辺製薬編)
4. Fukusumi, K.: Yukagaku, **12**, 93(1963).
5. Aoyama, S., Iwasaki, M.: Japan Heart J., **6**, 128(1968).
6. Iwagami, M.: Nagoya J. Med. Sci., **28**, 50(1965).
7. Nishigaki, I.: Vitamins(Kyoto), **37**, 617(1968).
8. Hirai, S. and Yoshikawa, M.: Internat'l. Sym. on Vit. E.(Hakone), p.228(1969).
9. Yoshikawa, M. and Hirai, S.: J. Gerontol., **22**, 162(1967).
10. 大沢仲昭, 井林博：ホルモンと臨床, **17**, 42(1969).
11. 平井俊策：日本臨床, **32(1)**, 8(1974).
12. Wolf, A. and Pappenheimer, A. M.: J. Neuropathol. Exp. Neurol.; **4**, 402(1945).
13. Goldschmidt, S. and Renn, K.: Chem. Ber., 55B, 628.
14. Kalish, G. H. and Lizio, D. N. R.: Science, **152**, 1390(1966).
15. Masugi, F. and Nakamura, T.: Vitamins (Japans) **51(1)**, 21(1977).
16. Blois, M. S.: Nature(London), **181**, 1199 (1958).