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Isolation and Identification of Free Phenolic Acids in Korean Ginseng

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Abstract

A method for isolation of some phenolic acids from Korean ginseng (*Panax ginseng* C.A.Meyer) was studied using silicic acid column chromatography, preparative thin layer chromatography and high performance liquid chromatography. Two phenolic compounds were isolated and identified as ferulic acid, mp 156-157°C and vanillic acid, mp 154-156°C by spectral data of Mass and NMR spectroscopy.

Introduction

The chemical, biochemical and pharmacological studies of Korean ginseng (*Panax ginseng* C.A.Meyer) have been mainly concentrated on ginseng saponin (ginsenoside) as effective component since the saponin mixture had been first isolated from American ginseng (*Panax quinquefolium*) in 1854[1]. The chemical structures of ginsenosides have been continuously elucidated. In parallel, various and controversial pharmacological effects of the ginsenosides used as saponin fraction or partially purified ones were reported and reconfirmed by many scientists at home and abroad during the past decades, that is, the stimulant action of ginsenoside Rg1 and depressant action of Rb1 on central nervous system,[2] antistress[3] and antifatigue action[4], promotion of DNA, RNA and protein synthesis[5-7] and so on. Recently, in view of the fact that the biological antioxidant reduces the lipid peroxide in cells known as the precurser of the lipofusin pigment concerned with the cellular aging,[8] an ether soluble acidic fraction of Korean ginseng that might contain antioxidative components was studied.[9] The fraction exhibited potent antioxidant activity against lipid peroxidation in liver from ethanol-intoxicated mice in contrast to ginsenosides such as Rb1, Rc, Rd and Rg1. And as active principles, maltol, salicylic acid and vanillic acid were isolated by using silica gel as an adsorbent.[10] Silica gel seemed to have no adequate selectivity for the separation of intact phenolic compounds in respect that they were derivatized in the procedure to get the pure components. In the present study, silicic acid instead of silica gel was used in order to improve the column selectivity in the fractionation of phenolic extracts. And using with semi-preparative high performance liquid chromatography phenolic components were isolated efficiently without derivatization. And also this study was conducted to investigate further the constituents in the phenolic fraction that had showed many unidentified components according to our previous study.[11]

Materials and Methods

Materials

The ginseng used in this study was white ginseng, dried 6-year-old fresh ginseng root, that was obtained from Jungpyong Exp. Station, Jungpyong, Korea. Reference phenolic acids, vanillic acid and ferulic acid, were obtained commercially and found to be chromatographically pure (TLC, HPLC).

Extraction and fractionation

Twenty two kilograms of dried ginseng root were extracted 3 times with 80 *L* of hot methanol (75°C) in a water bath. After filtration, the combined filtrates were evaporated to dryness under vacuum to give 4.3*kg* of syrupy extracts. The extracts were dissolved in 10 *L* of water and then fractionated according to the procedure shown in Fig. 1. The aqueous suspension was extracted with diethyl ether (4 x 10 *L*) and evaporated to dryness to give 340 *g* of extracts. The ether concentrates were dissolved in 4 *L* of diethyl ether and then extracted with 2-N HCl (2 × 2 *L*) and followed by the extraction with 5%-$NaHCO_3$ (2 × 2 *L*) and 5% NaOH (3 × 2 *L*) subsequently. The resulting 5% NaOH solution was acidified to pH 2 with 6-N HCl. After saturated with NaCl and extracted with diethyl ether (3 × 6 *L*), finally the ether solution was dried over anhydrous sodium sulfate, filtered and evaporated to dryness to give 7.2 *g* of phenolic extracts. The phenolic extracts were dissolved in 200 *ml* of 2% MeOH/$CHCl_3$ *(v/v)* in preparation for silicic acid column

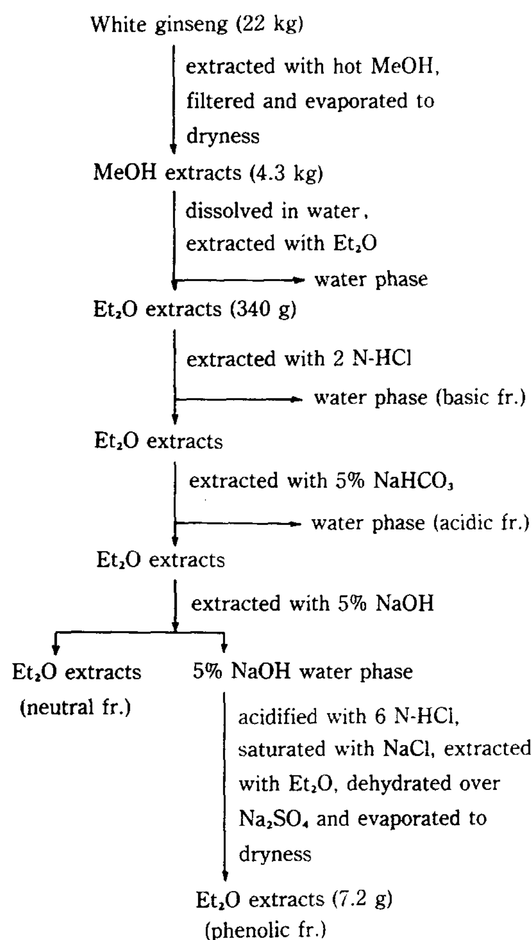

Fig. 1. Fractionation of MeOH extracts of white ginseng radix

chromatography.

Column chromatography

The column (2.5 *cm* inside diameter × 20 *cm*) was packed with silicic acid (100-300 mesh) in 2% $MeOH/CHCl_3$ *(v/v)*. Five *ml* aliquots of the phenolic extracts were introduced onto the column. Solvent flow was 4 *ml/min* and 20 *ml* fractions were collected. The following solvent system was used: 2% $MeOH/CHCl_3$ *(v/v)* up to 15 fractions, then programmed to 5% $MeOH/CHCl_3$. The end of the solvent program was 60% $MeOH/CHCl_3$ to wash the column. Eluted fractions were monitered at 325 *nm* and pooled into subfractions P-1, P-2, and P-3. Each fraction was evaporated to yield 0.45 *g*, 0.61 *g* and 0.64 *g*, respectively.

Preparative thin layer chromatography

Before the isolation of phenolic components with high performance liquid chromatography, the phenolic subfraction obtained above was purified, if necessary, with thin layer chromatography to enhance the efficiency in the HPLC system. Silica gel thin layer plate (20 × 20 *cm*, 0.75 *cm* thickness) was used with the developing solvent of 3% MeOH, 2% $HOAc/CHCl_3$ *(v/v)*. The bands were located by examination under UV light, eluted with methanol and subjected to final isolation by HPLC.

Chromatogenic reagents:

(a) Ferric chloride-Potassium ferricyanide: This reagent consists of equal volumes of 0.5% ferric chloride and 0.5% potassium ferricyanide. The chromatogram was sprayed with freshly prepared reagent and then with 2% HCl subsequently. Phenolic compounds produce blue spots. (b) DPNA(diazotized p-nitroaniline): This reagent was prepared by mixing three reagents; 0.5% p-nitroaniline in 2-N HCl, 5% $NaNO_2$, and 20% sodium acetate (1:10:30) in an ice bath. Characteristic yellow or purple colors were produced by phenolic components.

Isolation of phenolic components by high performance liquid chromatography

The isolation of phenolic components from the subfraction was performed by semi-preparative HPLC. The column was a 7.8 *mm* i.d. × 30 *cm* μ-Bondapak C_{18} reverse phase column. Solvent gradients were formed by a set of solvent programmer-controlled pumps. Solvents consisted of 86:8:6*(v/v)* water: acetonitrile: acetic acid (solvent A) and acetonitrile (solvent B). Solvent A as an initial condition was held for 10 *min* and then the gradients were formed to contain from 0 to 60% of solvent B for 40 *min*. Solvent flow was 1.5 *ml/min*. Sample volumn was 80-90μl and the phenolic components in the eluent were monitered at 325 *nm*.

Instrumental analysis

High performance liquid chromatographical system are as follows:

Pump, Waters model M-6000 A (Waters Assoc., Milford, Mass., U.S.A); injector, Waters model U6K; detector, model 450 Variable Wavelength Detector. UV spectral curves were measured with Shimadzu model 200 S spectrophotometer using 1 *cm* cuvettes in ethanol. Substituent effects were checked by adding two drops of 3%

aluminum chloride and then three drops of 1% aqueous sodium hydroxide to cause a shift in peaks. Infrared spectra were measured by Perkin-Elmer model 599 B spectrophotometer. Mass spectra were taken at 75 *eV* on Hewlett Packard model 5985 spectrometer. Proton NMR spectra were obtained in acetone-d_6 and DMSO-d_6 solution at 80 MHz using TMS as internal standard on Varian FT-80A spectrometer. And melting points were measured by Fisher-Johns melting point apparatus (Fisher Scientific Company, USA).

Results and Discussion

Elution of the phenolic extracts obtained by the procedure shown in Fig. 1 with 2% MeOH/$CHCl_3$ and 5% MeOH/$CHCl_3$ on silicic acid column gave the resulting elution curve shown in Fig. 2. Based on the elution curve, appropriate fractions were combined to give fraction P-1 from fraction 5 to 9, P-2 from fraction 10 to 14 and P-3 from fraction 19 to 24 and evaporated to give 0.45 *g* of P-1, 0.61 *g* of P-2 and 0.64 g of P-3, respectively. Among these three fractions P-2 was further investigated for the isolation of phenolic compounds. And the remaining two fractions were reserved for another studies. Prior to the isolation with HPLC, P-2 was purified by preparative thin layer chromatography as shown in Fig. 3. When visualized under UV light (254 *nm*) and sprayed with $FeCl_3/K_3Fe(CN)_6$ and diazotized p-nitroaniline, P-2-2 was the major band that showed the darkest UV quenching and the most intensive color reactions. Band P-2-2 was eluted with methanol, filtered and evaporated to appropriate concentration for HPLC.

Finally, the isolation of pure phenolic compounds was performed with the purified fraction P-2-2 by HPLC. Fig.4 shows the HPLC chromatogram of the fraction P-2-2. Five major peaks were collected from the chromatograph in

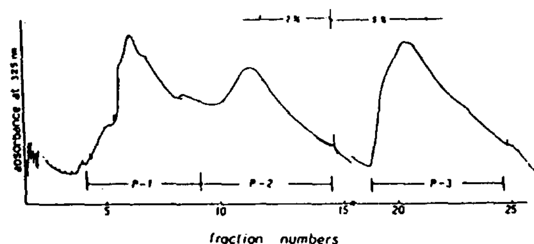

Fig. 2. Separation of phenolic acid extracts on silicic acid column (2.5 *cm* i.d. × 20 *cm*) using 2% MeOH/$CHCl_3$ and 5% MeOH/$CHCl_3$ at 4 *ml/min*

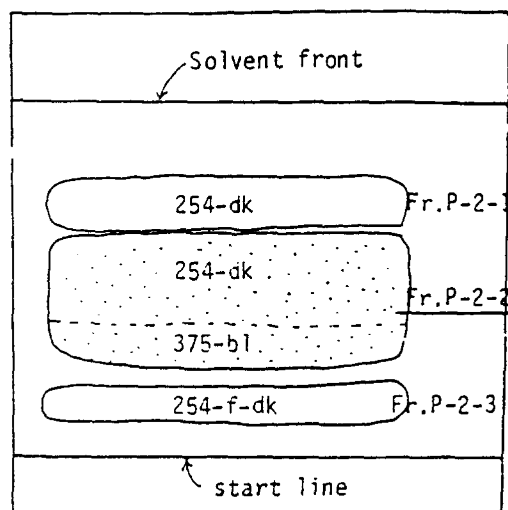

Fig. 3. Preparative silica gel TLC chromatogram of the subfraction P-2 in the developing solvent of 3% MeOH, 2% HOAc/$CHCl_3$
***254-dk;dark at 254 nm, bl;blue, f;faint**

evaporating round flask and evaporated to dryness. Dried residues of both peak P-2-2-2 and peak P-2-2-4 were white powder with some impurities unlike the other peaks. The residue of peak P-2-2-2 was recrystallized from water to give white crystal of 5 *mg*, mp. 154-156°C, and designated as compound I. The residue of peak P-2-2-4 was also recrystallized from water to give white needle crystal of 12 *mg*, mp 156-157°C, and designated as compound II.

Identification of compound I (vanillic acid)

Compound I showed a positive reaction with $FeCl_3/K_3Fe(CN)_6$ and diazotized p-nitroaniline to give blue

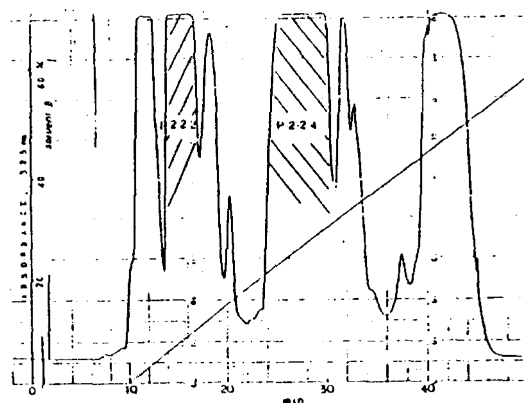

Fig. 4. HPLC preparative column separation of the subfraction P-2-2 using μ-Bondapak C_{18} column

and yellow spots on TLC plate, having the Rf value of 0.32 in the developing solvent of 3% MeOH, 2% HOAc/$CHCl_3$ *(v/v)*. When visualized under short wave(254 *nm*) UV light, it appeared as dark spot and had no fluorescence under long wave(375 *nm*). The UV spectrum showed the absorption maximum at 260 nm (ε; 5042) and 290 *nm* (ε; 3673), which were shifted to 268*nm* and 295 *nm* by the addition of aluminum chloride, indicating that the compound has a phenolic group. Further addition of sodium hydroxide also caused the shift to higher wavelength of the absorption maximum by resulting in phenolate ions. IR spectrum shows absorption bands at 3460 cm^{-1} (hydroxyl group), 1670 cm^{-1} (carboxyl group), 1590 cm^{-1} (aromatic double bond) and 1200 cm^{-1} (ether bond). Mass spectrum (Fig. 5) shows molecular ion at *m/e* 168, M^+-CH_3 at *m/e* 153, M^+-CH_3CO at *m/e* 125, and M^+-CH_3CO-CO at *m/e* 97. The proton NMR spectrum of compound I in acetone-d_6 (Fig. 6) showed the presence of three aromatic protons with one methoxyl group. The sharp singlet at 3.87 *ppm* integrating for three protons was attributed to one methoxyl group at C-3 position. The three aromatic protons give two doublets (J = 8 cps) at 7.55 *ppm* (6-H) and 6.85 *ppm* (5-H) and one singlet at 7.52 *ppm* (2-H) buried under the signal for 6-H. As the chemical shift of hydroxyl group proton was not found in this spectrum using acetone-d_6, the solvent was replaced with DMSO-d_6. As a result, a broad singlet at 9.72 *ppm* integrating for one proton emerged and assigned to the proton of the hydroxyl group. From the spectroscopical findings, compound I was identified as vanillic acid (4-hydroxy-3-methoxy benzoic acid).

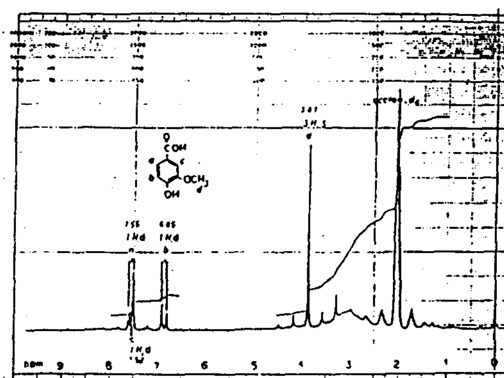

Fig. 6. ¹H-NMR spectrum of compound I

Identification of compound II (ferulic acid)

Compound II also showed a positive reactions with $FeCl_3/K_3Fe(CN)_6$ and diazotized p-nitroaniline to give blue and purple spot on TLC plate. The Rf value was 0.40 in the developing solvent of toluene: ethylacetate: formic acid (5:4:1), showing the same value as that of the authentic standard of ferulic acid. When visualized under short wave (254 *nm*) UV light, it appeared as dark spot and showed blue fluorescence under long wave (375 *nm*). UV absorption maximum was at 322 *nm* (ε; 6038) and was shifted to 334 nm by the addition of aluminum chloride, and further shifted to 348 nm by sodium hydroxide, showing the presence of a phenolic group. The IR spectrum showed the presence of hydroxyl group (3440 cm^{-1}), carboxyl group (1660 cm^{-1}), aromatic double bond (1600 cm^{-1}), ether bond (1200 cm^{-1}), and olefinic bond (1610 cm^{-1}). The IR band at 1610 cm^{-1} was not found in the spectrum of compound I. Mass spectrum (Fig. 7) showed molecular ion at *m/e* 194, M^+-CH_3 at *m/e* 179, M^+-COH, OH at *m/e* 148, *m/e* 148 $-CH_3$ at *m/e* 133 and *m/e* 133 $-$CO at *m/e* 105. In the proton NMR spectrum of compound II (Fig. 8) two olefinic protons and three aromatic protons were observed along with three protons of a methoxy group. Two olefinic protons give two doublets (J = 16 cps) at 7.55 *ppm* ($\underline{CH}$=CH-COOH) and 6.31 *ppm* (CH=$\underline{CH}$-COOH). The doublet at 7.26 *ppm* was considered to be due to a C-2 proton that interacts with the C-6 proton. The two doublets (J = 8 cps) at 7.07 *ppm* and 6.82 *ppm*, integrating

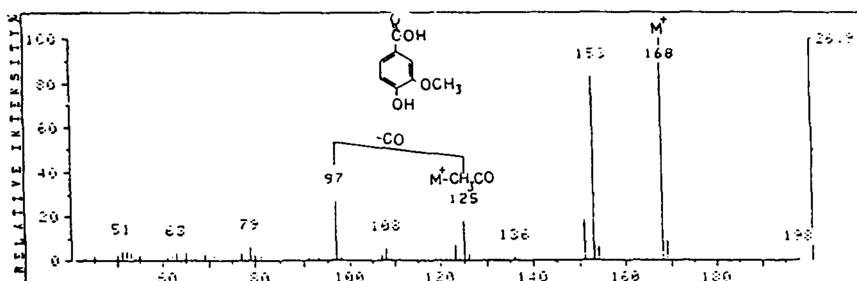

Fig. 5. Mass spectrum of compound I

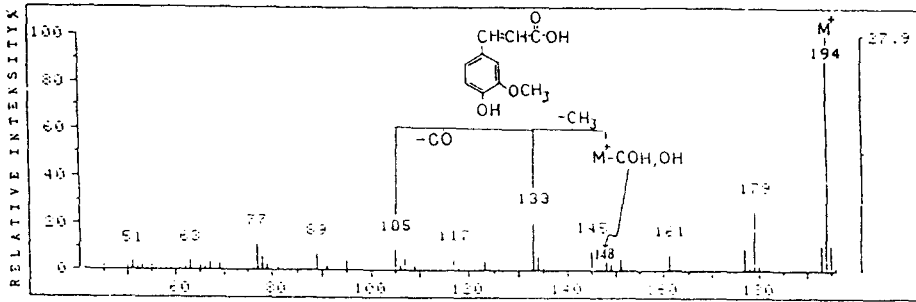

Fig. 7. Mass spectrum of compound II

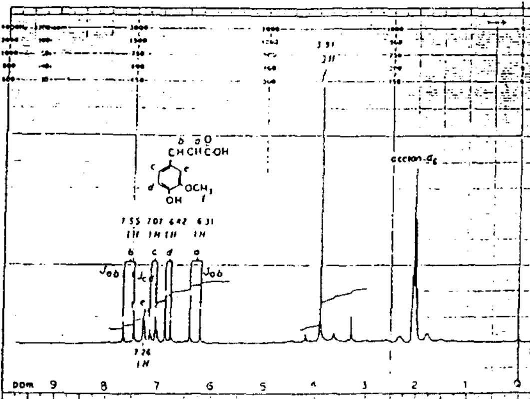

Fig. 8. ¹H-NMR spectrum of compound II

one proton, respectively, were assigned to the protons at C-6 and C-5 positions. The doublet at 7.07 *ppm* appeared as doublet of doublet due to the interaction between C-6 proton and C-2 proton. One methoxy group at C-3 position gave a singlet at 3.91 *ppm* integrating three protons. According to these properties found above, compound II was concluded to be ferulic acid (4-hydroxy-3-methoxy cinnamic acid).

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인삼의 유리 페놀성 분획중 phenolic acid의 순수분리 동정

김만욱 · 위재준 · 박종대

한국인삼연초연구소

한국백삼의 유리페놀성 분획(free phenolic acid fr.)에서 phenolic acid의 분리 정제방법을 검토하였다. 먼저 silicic acid column에 의해 소분획으로 나누고 prep-TLC 및 HPLC하여 정색반응 및 Rf치에 의해 phenolic acid로 추정되는 두개의 화합물을 순수 분리하고, 결정을 얻어 UV, IR, MS, NMR로 그 구조를 밝힌 결과 ferulic acid와 vanillic acid로 확인되었다.