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Identification of Antimutagenic Compound from Kale by High Performance Liquid Chromatography and Mass Spectrometry

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Abstract

Kale (*Brassica oleracea* var. *acephala*) is one of *Cruciferous* vegetables that is closely related to the wild ancestral form of cabbage. The methanol extract of kale which contains the active compound(s) under *Salmonella* assay system was fractionated with chloroform to collect the nonpolar solvent soluble compounds, and then further fractionation was carried out by silica gel column chromatography. Among kale extracts separated by silica gel column chromatography, the fractions of 4, 5 and 6 exhibited strong antimutagenic activities. The major active compounds from the fraction were identified as chlorophyll derivatives by the analysis with HPLC-frit-MS. The molecular weights of each chlorophyll derivatives in the sample were acquired from the peaks of positive ion atmosphere pressure chemical ionization (APCI) mass spectrometry.

Key words: kale, antimutagenicity, HPLC-MS, chlorophyll

INTRODUCTION

Kale (*Brassica oleracea* var. *acephala* DC) which came from Asia Minor is closely related to the wild ancestral form of cabbage (1). Kale have a rich source of vitamins and minerals, particularly vitamin C (146 mg/100 g), vitamin U (54 mg/100 g), β-carotene (70.3 μg/g) and calcium (18 mg/100 g) (2,3). Also, kale has much more chlorophyll content (187 mg/100 g) than that of spinach (127 mg/100 g) which was generally known as containing a lot of chlorophyll. As a result of quantitative analysis of chlorophyll components, the content of chlorophyll a, chlorophyll b, pheophytin a and pheophytin b in kale was determined to be 137 mg/100 g, 46.4 mg/100 g, 1.69 mg/100 g and 1.7 mg/100 g, respectively (4).

As the methods of chlorophyll analysis have developed considerably, the analysis of mass spectra of chlorophyll have been acquired by using several techniques (5-13). In recent mass spectrometry (MS), the atmospheric pressure ionization (API) technique that ionizes a molecule under atmospheric pressure rather than the conventional vacuum condition in a mass spectrometer is rapidly growing (14). Atmospheric pressure chemical ionization (APCI) uses high voltage corona discharge in the ion source and is one of the soft ionization methods in mass spectrometry (15). This method is also useful for the on-line interfacing of a mass spectrometer into a liquid chromatographic (LC) system under atmospheric pressure. The application of the LC/API/MS system showed the fast and sensitive way for the analysis of various samples in polar and involatile nature. Moreover, the sensitivity and selectivity for the identification of different kinds of molecules can be enhanced by the use of selected ion monitoring.

Chlorophyll has appeared to suppress genotoxicity of 3-amino-1-methyl-5H-pyrido [4,3-b] indole (Trp-P-2) on *Drosophila melanogaster* (16). Chlorophyll and copper chlorophyllin have also been shown to be effective in inhibiting mutagenesis induced by the carcinogens 3-methylcholanthrene and benzo[a] pyrene using the Ames *Salmonella*/microsome activation system (17,18). Chlorophyll a and b were far less active than the copper chlorophyllin and hemin in blocking mutagenesis. The antimutagenic activities of a variety of common vegetables was found to correlate with the chlorophyll content of the vegetables (19).

The objective of this study was to extract, separate and identify the antimutagenic compounds from kale, one of the cruciferous vegetables. The methanol extract of kale was fractionated with chloroform and then further fractionation was carried out by silica gel column chromatography. The active antimutagenic fractions (fr. 4, 5 and 6) were collected by silica gel chromatography. The separation and struture analysis of active compound(s) were performed using a triple quadropole LC/MS with API mode.

MATERIALS AND METHODS

Preparation of sample

Fresh kale was purchased from a local market in the city of Pusan, Korea. Kale was washed, drained, chopped, freeze-dried and homogenized. A dried sample was extracted with methanol (3 times with 20 ml portions/g sample). The methanol extract of the sample was condensed by a vacuum evaporator (Buchi 011 & 461, Switzerland) (20).

Fractionation of sample

The condensed sample was suspended by $CHCl_3$:Methanol:

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H_2O (10:1:9, v/v/v). The chloroform fraction obtained was condensed and the further fractionation was carried out by silica gel column chromatography. The silica gel (Kiesel gel Art. 7734, Merck) was suspended and added to a column (100 cm×5 cm i.d.). The sample mixed with silica gel (10 g) was applied to the column equilibrated with chloroform:methanol:H_2O (65:35:10, v/v/v). The eluates were collected in aliquots of 50 ml per tube (fractions 1 to 26) (21,22).

Ames mutagenicity test

Salmonella typhimurium TA100, a histidine requiring mutant, was provided by Dr. B.N. Ames, University of California (Berkeley), CA and was maintained as described by Maron and Ames (23). Aflatoxin B_1 (AFB_1) was purchased from Sigma Chemical Co. and dissolved in dimethyl sulfoxide (DMSO). The genotype tester strain was routinely checked for its histidine requirement, deep rough (*rfa*) character, UV sensitivity (*uvrB* mutation) and the presence of R factor.

A modified plate incorporation test (24) in which 30 min liquid preincubation of the organism with the test compound was employed to determine the antimutagenic effects of kale on the mutagenesis of aflatoxin B_1 (AFB_1). In the preincubation test, 0.5 ml of S9 mix. was distributed in sterile capped tubes in ice bath and then 0.1 ml of testers from overnight culture ($1\sim2\times10^9$ cells/ml) and 0.1 ml of test compound (50 μl of mutagen and 50 μl of sample) were added. The tubes were gently vortexed and preincubated at 37°C for 30 min, and 2 ml of the top agar kept at 45°C was added to each tube and vortexed for 3 seconds. The resulting entire mixture was overlaid on the minimal agar plate. The plates were incubated at 37°C for 48 hrs and then the revertant bacterial colonies on each plate were counted.

Dose response tests of the mutagens on the tester strains were carried out to determine the regions of revealing mutagenicity induced by the mutagens. Toxicity tests for each sample were also carried out. The samples employed for the antimutagenic test in this study did not show any toxicity to the tester strains.

Identification of HPLC-Mass

Among the collected fractions, the fraction 4, 5 and 6 were condensed and dissolved in $CHCl_3$. Each 10 μl sample solution was injected into a 2.1×200 mm Hewlett Packard (California, USA) 5 μm Hypersil ODS column for the separation at 0.5 ml/min of the mobile phase using HP Model 1050 HPLC quaternary pump system and analyzed by HPLC-MS. The end of the column was connected through a UG Biotech (Manchester, UK) LC/MS interface into a UG Quattro triple quadropole mass spectrometer. The positive ion APCI (atmosphere pressure chemical ionization) mass spectra were obtained by using the APCI method with a UG Quattro triple quadropole mass spectrometer (UK). The precusor ions formed by APCI ionization were fragmented by collisional activation using N_2 in the collision cell. The API source was maintained at the following conditions; discharge voltage at 3 kV, nitrogen as a curtain gas, analyzer pressure at 1×10^{-6} mbar, source pressure at 1×10^{-5} mbar. The corona discharge ion source was used with a heated nebulizer probe. The API source and probe temperature were maintained at 120°C and 450°C, respectively. Ions were sampled from atmospheric pressure into the mass analyzer *via* an intermediate pressure region defined by 20 V between sampling and skimmer plate. Data acquisition and instrument control were achieved by MassLynx data system with Digital 66 MHz DEC80486 workstation. The operation condition HPLC-MS analysis were shown in Table 1.

RESULTS AND DISCUSSION

After the chloroform fraction of kale was separated by silica gel column chromatography, the fr. 4, 5 and 6 were detected as the most strongly antimutagenic fractions on AFB_1 induced mutagenesis (Fig. 1). In the case of *Salmonella typhimurium* TA100 with the metabolic activating system, the mutagenesis of AFB_1 is reduced to 82.1%, 78.9% and 68.8% by adding 50 μl of fr. 4, 5 and 6 per plate, respectively. In the case of

Table 1. The operation condition of HPLC-frit-APCI mass analysis

Instrument	: UG Quattro Triple Quadropole Mass Spectrometer (UK)
Method	: The atmosphere pressure chemical ionization (APCI)
Column	: Hewlett Packard 5 μm Hypersil ODS column (2.1×200 mm)
Corona discharge	: 3000 volts
N_2 Nebulizev	: 200 L/hr
Solvent	: Methanol
Flow rate	: 0.5 ml/min

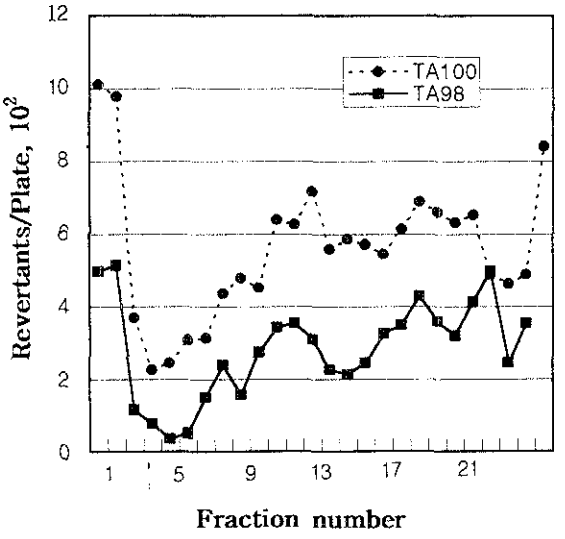

Fig. 1. Revertant numbers obtained from various fractions from chloroform fraction of methanolic extract of kale on the mutagenicity induced by aflatoxin B_1 (AFB_1, 0.28μg/plate) in *Salmonella typhimurium* TA100 and TA98. *The spontaneous number and the revertants from aflatoxin B_1 in TA100 and TA98 were 115±4 ; 736±49 and 21±2 ; 412±32, respectively.

Salmonella typhimurium TA98 with the metabolic activating system, the mutagenesis of AFB_1 is inhibited to 85.7%, 96.2% and 92.3% by adding 100 μl of fr. 4, 5 and 6 per plate, respectively. Our experiment confirmed the fact that the mutagenicities of AFB_1, indirect carcinogen were inhibited in the presence of fr.4~6.

As shown in Fig. 2, the chlorophyll a and b were analysed to have a number of chlorophyll derivatives such as pheophytin a, pheophytin b, chlorophyllide a, chlorophyllide b, pheophorbide a, pheophorbide b, pyropheophytin a and pyropheophytin b. Van Breemen et al. (25,26) reported that the chlorophyll a, pheophytin a, chlorophyllide a, pheophorbide a, pyropheophytin a, chlorophyll b, pheophytin b, chlorophyllide b, pheophorbide b and pyropheophytin b have Mw. 892.5, 870.8, 614.3, 592.1, 812.5, 906.5, 884.5, 628.2, 606.1 and 826.3, respectively, through analysis by HPLC-frit-fast atom bombardment-MS. Chlorophyll a is contains a CH_3 group in the R portion of chlorophyll, the chlorophyll b is contains a CHO group in R portion of chlorophyll, the pheophytin a and pheophytin b are produced by removal of Mg (Mw. 22) from chlorophyll, the chlorophyllide a and chlorophyllide b are produced by removal of phytyl chain (Mw. 278) from chlorophyll, the pheophorbide a and pheophorbide b are produced by removal of phytyl chain and Mg from chlorophyll, and the pyropheophytin a and pyropheophytin b are produced by removal of Mg and $COOCH_3$ group from chlorophyll (25).

As the mixtures of fr. 4, 5 and 6 were injected into HPLC-frit-MS, the total ion chromatogram of five main peaks is shown in Fig. 3. Also, the APCI mass spectra of five main peaks from the HPLC chromatograms are shown in Fig. 4 and are assumed to be chlorophyll derivatives. The *m/z* 613.4 [chlorophyllide a - H] peak of mass spectrum (Fig. 4A) which is analysed in retention time 0.75 min of HPLC chromatogram (Fig. 3) is assumed to be chlorophyllide a (Mw. 614.3) and the *m/z* 591.5 peak[pheophorbides a-H] as pheophorbides a (Mw. 592.1). The *m/z* 613.5 peak[chlorophyllide a-H] of mass spectrum (Fig. 4B) which is analysed in retention time 1.32 min is assumed to be chlorophyllide a (Mw. 614) and the *m/z* 585.5 peak is assumed to be formed by removal of CHO, CH_2 and phytyl chain from chlorophyll b (Mw. 906.5). The *m/z* 585.4 peak of mass spectrum which is analysed (Fig. 4C)

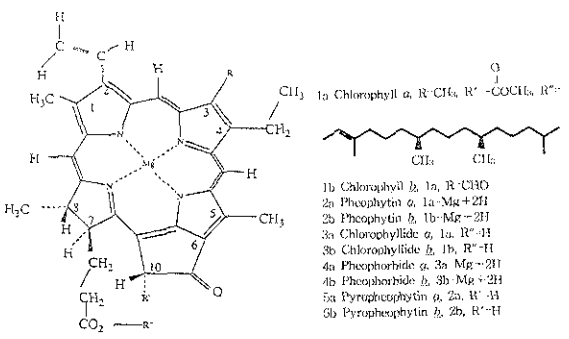

Fig. 2. Structure of chlorophylls and chlorophyll derivatives.

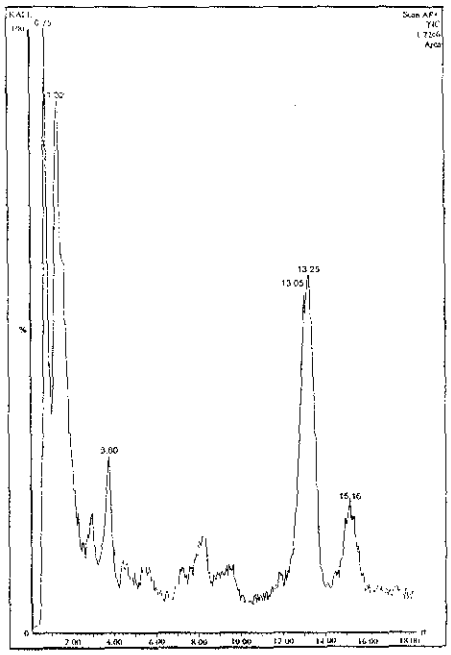

Fig. 3. Total-ion chromatogram using positive ion HPLC-frit-APCI Mass of the antimutagenic fraction (mixtures of fr. 4, 5 and 6) from kale.

in retention time 3.80 min is assumed to be formed by the removal of CHO, CH_2 and phytyl chain from chlorophyll b (Mw. 906.5). The *m/z* 894.1 peak [chlorophyll a+2H] of mass spectrum (Fig. 4D) which is analysed in retention time 13.25 min is assumed to be chlorophyll a (MW. 892.5), the *m/z* 871.3 peak of mass spectrum is assumed to be pheophytin a (Mw. 870.8), and the *m/z* 517.3 peak of mass spectrum is assumed to be formed by the removal of $COOCH_3$ and CH_3 from pheophorbide a (Mw. 592.1). The *m/z* 893.6 peak [chlorophyll a+H] of mass spectrum (Fig. 4E) which is analysed in retention time 15.16 min is assumed to be chlorophyll a (Mw. 892.5), the *m/z* 871.3 peak of mass spectrum is assumed to be pheophytin a (Mw. 870.8), and the *m/z* 517.8 peak of mass spectrum is assumed to be formed by the removal of $COOCH_3$ and CH_3 from pheophorbide a (Mw. 592.1). These fragmentation patterns are similar to the results obtained from the standard chlorophyll a (data not shown).

Because of the extraction and fractionation process, chlorophyll derivatives were detected as the degraded condition without phytol or Mg^{2+} or side chain from chlorophyll. The phytol and Mg^{2+} in the chlorophyll structure are unstable and are readily degraded, but this change did not affect the fundamental structure of porphyrin. The antimutagenic effect of chlorophyll was not affected in Mg^{2+} removal or acidic condition, and was influenced by open chain tetrapyrrole in relation to porphyrin structure in chlorophyll. This is analogus to its role as the antimutagenic agent of plant phenolic compounds (18). As a result of analysis with LC-MS, the antimutagenic compounds separated from the chloroform extract of kale were

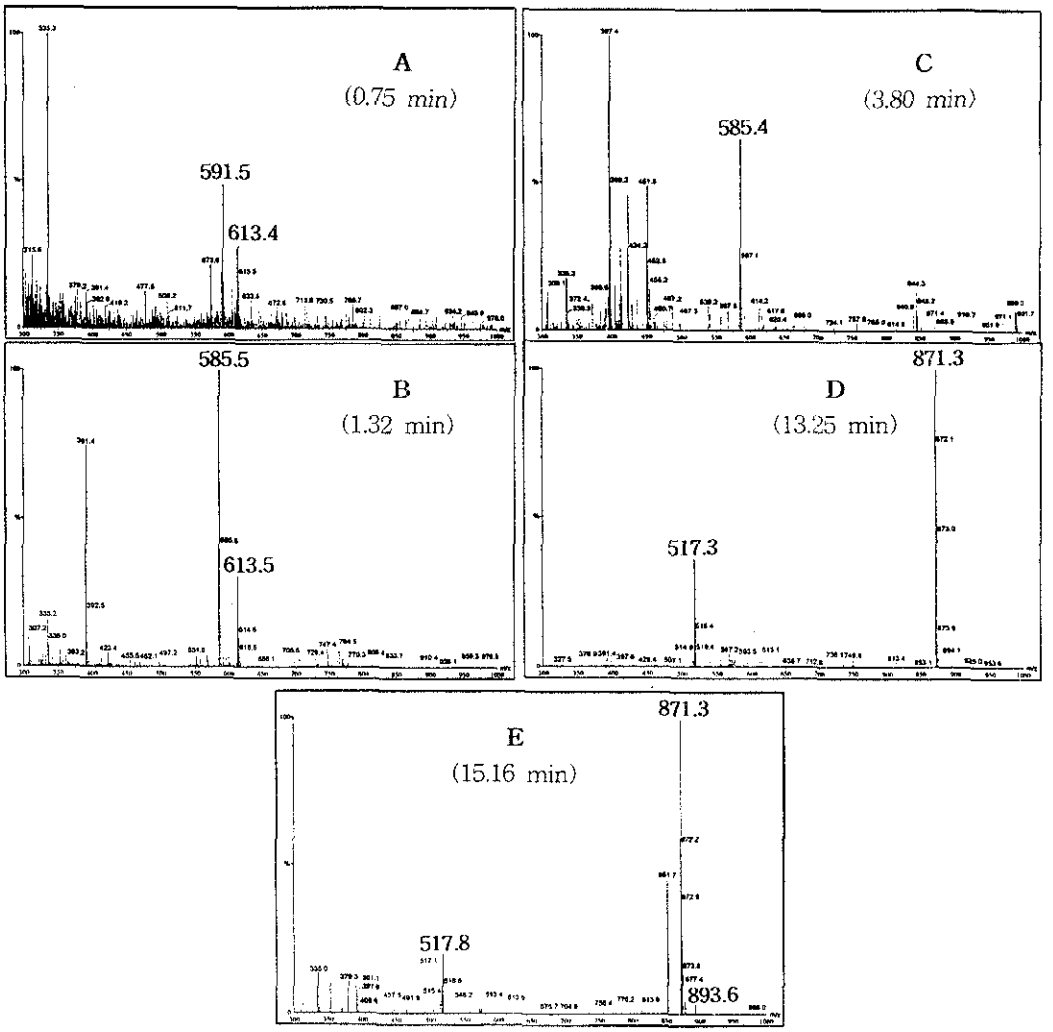

Fig. 4. Mass spectrometric analysis of five main peaks from the HPLC-frit-APCI mass chromatograms shown in Fig. 3. A: APCI mass spectrum at a retention time of 0.75 min, B: APCI mass spectrum at a retention time of 1.32 min, C: APCI mass spectrum at a retention time of 3.80 min, D: APCI mass spectrum at a retention time of 13.25 min, E: APCI mass spectrum at a retention time of 15.16 min

identified as chlorophyll derivatives.

In physiological aspects, chlorophyll ingested in human food is essentially not absorbed. The magnesium in chlorophyll is removed by stomach acid to yield a protoporphyrin, the phytyl ester may be partly removed by digestive enzymes, and the remainder is recovered in the feces as pheophytin. The chemical changes of chlorophyll which are induced by the monogastric mammalian digestive system produce a molecule more closely resembling chlorophyllin. This suggests that the digestive processes may play a part in activating the ingested chlorophyll to be an effective blocker of mutagenesis within the lumen of the lower gastrointestinal tract (18). The pheophytin a as compound with antimutagenic activity was identified from Savoy Chieftain cabbage. This pheophytin a belongs to a class of compounds that are produced by removal of Mg^{2+} from chlorophyll and inhibit the mutagenicity of a variety of mutagens. Chlorophyll a and b inhibited the mutagenicity of 3-MC, benzo [a]pyrene (BP) and cigarette smoke condensate, and extracts of dietary mixtures. The chlorophyll was significantly more active than pheophytin against the mutagenicity of 2-aminoanthracene (2-AA) and N-methyl nitrosourea (MNU) (27). Typically, the most abundant fragment ion in the mass spectra of chlorophyll derivatives, the formation of $[M-278]^+$ ions, is indicative of the presence of the phytyl chain (25,26). The active antimutagenic compounds in Kale were identified as phytol by the GC-MS (28). The analysis of compounds by GC-MS is possible to below Mw. 500. Because of large molecular weight, chlorophyll a (Mw. 892.5) and chlorophyll b (Mw. 906.5) didn't seem to be detected by the mass spectrum of GC-MS. To confirm the chlorophyll derivatives as the active compounds in kale, we analysed using HPLC-MS, which is able to detect large molecular weights. Therefore, phytol detected by GC-MS is thought to be a compound which is separated from chlorophyll during the extracting and fraction-

ating process (21).

CONCLUSIONS

After the chloroform fraction of kale was separated by silica gel column chromatography, the fr. 4, 5 and 6 were detected as the strongest antimutagenic fractions in the Ames assay. The total ion chromatogram of active compounds generated from HPLC-frit-MS analysis had five main peaks. The mass spectra of main peaks in the HPLC chromatogram were assumed to be chlorophyll a, pheophytin a, chlorophyllide a, chlorophyll b without a CHO, CH_2 and phytyl chain, pheophorbide a, and pheophorbide a without a $COOCH_3$ and CH_3 group, respectively. As a result, the antimutagenic compound(s) in kale were identified as chlorophyll derivatives. We will have further studies regarding the relationships between various chlorophyll derivatives and their antimutagenic (anticancer) effects.

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