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Effect of High Speed Drying on Antioxidant Properties of Enzymatic Digests from Citrus By-products and Their Protective Effect on DNA Damage Induced by H_2O_2

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Abstract The aim of this study was to evaluate the antioxidant activities of enzymatic digests from citrus by-products (CBPs) prepared by high speed drying (HSD). HSD needs a short time (60 min) for drying and can be used in a commercial scale. Enzymatic digests were prepared from the CBPs using 6 enzymes such as aminoglucosidase (AMG), celluclast, pectinex, termamyl, ultraflo, and viscozyme. Antioxidant activities of AMG digest from CBPs were evaluated by different *in vitro* models such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, alkyl, H_2O_2 scavenging, metal chelating, lipid peroxidation, and comet assays, and exhibited strong activities. The antioxidant compounds were detected by an high performance liquid chromatography (HPLC) coupled on-line to an 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) scavenging detection system, and found that hesperidin was the key compound. Therefore, the results obtained in this study suggest that HSD is an effective method to transform wet CBPs into dried form, and CBPs are potential source of natural antioxidant.

Keywords: antioxidant activity, citrus by-product, enzymatic digest, high speed drying, HPLC coupled on-line to an $ABTS^+$

Introduction

Oxidation is an essential process in living organisms to produce necessary energy to fuel biological activities. However, over production of oxygen-centered free radicals and other reactive oxygen species (ROS) result in cell death and tissue damage. ROS has been implicated in several chronic diseases, including aging, cancer, cardiovascular diseases, and diabetes (1). Antioxidants are vital compounds which are capable of protecting the body from damage caused by free radicals induced oxidative stress.

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are largely employed as preservatives in pharmaceutical, cosmetic, and food industries. However, those synthetic antioxidants are believed to cause side effects on human (2,3). Hence, most consumers are concerned about the safety of their food and about the potential effects of synthetic additives on their health. Therefore, the need to replace these synthetic antioxidants with naturally-occurring antioxidants probably with safe antioxidants has initiated with the screening of plant sources, especially the inexpensive by-products from agricultural industries (4).

Huge amount of citrus by-products (CBPs) are formed every year from the citrus processing plants, and they are excellent sources of different phytochemicals (5-7), many of them may contain potent biological activities. As these by-products may be deteriorated rapidly they are needed to transform into dried form if they are needed to use in effective manner. In order to increase the efficacy of the drying process, high speed drying (HSD) which can be used in a commercial scale, was applied in this study. This method is more economical and less time consuming as it is able to transform large amount of by-products into dried form at a single turn. In this study efficacy of HSD for CBPs drying was examined by comparing with a conventional freeze drying because the final quality and potential activity of the dried by-products would be determined by the structural and compositional modifications which might have occurred during the drying treatment (8).

The extraction method of antioxidants affects the total polyphenolic contents and antioxidant capacities (9). Water, methanol, ethanol, and ethyl acetate are commonly used to extract bioactive compounds from plant materials. However, enzymes can macerate the tissues by breaking down of cell walls, and cellular components can be come out of the cells. In the present study, hence the efficiency of the enzymatic digest from CBPs dried by HSD and freeze drying (FD) was evaluated, by comparing the antioxidant activities with that of the water extracts.

The enzymatic digestion is a choice of interest to get higher yield of desired compounds from the natural resources while it is an easily accessible extraction and purification process in the industry (10-12). The enzymes can work primarily by macerating the tissues and breaking down the cell walls and complex interior storage materials to release the bioactive compounds. Furthermore, enzymatic digestion

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process possesses the number of advantages and characteristic features compared with the conventional extraction procedures. Higher extraction efficacy, water solubility, variation of constituents, minimized environmental pollution, and relatively less expensiveness are some of those advantages.

The aim of this study was to evaluate the antioxidant activities of enzymatic digests from CBPs prepared by HSD.

Materials and Methods

Materials 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrrolin *N*-oxide (DMPO), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH), and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Viscozyme L (a multi-enzyme complex containing wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase), Celluclast 1.5 L FG (catalyzing the breakdown of cellulose into glucose, cellobiose, and higher glucose polymers), AMG 300L (an exo-l,4-alpha-D-glucosidase), Termamyl 120 L (a heat stable α-amylase), Ultraflo L (a heat-stable multi-active β-glucanase), and Pectinex 5X L (catalyze the pectic polymers) were purchased from Novo Co. (Bagsvaed, Denmark). All other chemicals used were in analytical grade.

High speed drying (HSD) of citrus by-products HSD is a process that can be used to transform large amount of agricultural or processing by-products into a dried form within a short time effectively as an alternative method to existing drying methods. The principle behind HSD was described in our previous study (13).

Citrus by-products (CBPs) were obtained from the Jeju provincial development Company in Korea and they were stored at −50°C until drying with high speed drier (Okadora Korea, Incheon, Korea). The operating conditions of the HSD were as follows: drying time 90 min, heating temperature 120°C, and the amount of dried sample obtained from the drying process was 3 kg, and the operating conditions of freeze dryer (Ilshin Co., Seoul, Korea) were; drying time 3 days, heating temperature −56°C, and the amount of dried sample obtained from the drying process was about 600 g. Then, the dried CBPs were pulverized into fine powder using a grinder (MF 10 basic mill; GMBH & Co., Staufen, Germany) and sieved through a 300-mm standard testing sieve. The proximate chemical compositions were as follows. Moisture, ash, carbohydrate, fat, and protein; 8.51, 3.8, 75.69, 2.8, and 9.2%, respectively in CBPs dried by HSD, and 9.17, 3.75, 75.68, 1.6, and 9.8%, respectively in CBPs dried by freeze drying (FD).

Preparation of enzymatic digests Six enzymes, such as AMG, Celluclast, Pectinex, Termamyl, Ultraflo, and Viscozyme were used in the enzymatic digestion process. A 1 g of CBPs dried by HSD or FD was mixed with 100 mL of distilled water. The optimum pH of the each mixture was adjusted with 1 M HCl/NaOH. Thereafter, the enzymatic digestion was performed by the addition of enzyme (100 μL or 100 mg) for 24 hr according to the method described by Heo *et al.* (12). The resultant mixture was inactivated by keeping in a water bath at 100°C for 10 min and filtered. Finally, each digest was adjusted to pH 7.

Total polyphenolic content assay Total polyphenolic content was determined according to the method described by Chandler and Dodds (14). One mL of the digest was mixed in a test tube containing 1 mL of 95%(v/v) ethanol, 5 mL of distilled water, and 0.5 mL of 50%(v/v) Folin-Ciocalteu reagent. The resultant mixture was allowed to react for 5 min and 1 mL of 5%(w/v) Na_2CO_3 was added. It was mixed thoroughly and placed in a dark condition for 1 hr, and absorbance was recorded at 725 nm using ultraviolet (UV)-VIS spectrophotometer (Opron 3000; Hansan Tech. Co., Seoul, Korea). A gallic acid standard curve was obtained for the calculation of polyphenolic content.

Total flavonoid content assay The total flavonoid content of the digest was determined by the method of Zhuang *et al.* (15) with slight modifications. A 0.5 mL aliquot of the sample solution was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a 5%(w/v) $NaNO_2$ solution. After 6 min, 0.15 mL of a 10%(w/v) $AlCl_3$ solution was added and allowed to stand further 6 min. Thereafter 2 mL of 4%(w/v) NaOH solution was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL. Then the mixture was properly mixed and allowed to stand for 15 min. Absorbance of the mixture was taken at 510 nm. Rutin was used as standard for the quantification of total flavonoid.

DPPH radical scavenging assay This assay was based on the scavenging ability of stable DPPH radicals by the radical scavenging constituents in the digest. Method described by Nanjo *et al.* (16) was used to investigate the free radical scavenging activity in electron spin resonance (ESR) spectrometer. DPPH solution in MeOH was prepared at the concentration of 60 μM. A 60 μL fraction of the digest was added to the same volume of freshly prepared DPPH. Then the reactants were thoroughly mixed and transferred to 50-μL glass capillary tube and fitted into the ESR spectrometer. The spin adduct was measured after 2 min. The measurement conditions were as follows; central field 3,475 G, modulation width 0.8 mT, amplitude 500 mT, microwave power 5 mW, sweep width 10 mT, and temperature 298 K.

Hydroxyl radical (·OH) scavenging assay Ability of the enzymatic digest to scavenge the hydroxyl radical was investigated by the method described by Rosen and Rauckman (17). The Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow \cdot OH+OH^-$), a well known and defined generator of ·OH radicals was used, and the radicals produced were reacted rapidly with nitrone spin trap DMPO. The reaction mixture contained 20 μL digest, 20 μL of 0.3 M DMPO, 20 μL $FeSO_4 \cdot 7H_2O$, and 20 μL of 10 mM H_2O_2. The resultant DMPO-OH adducts was investigated, and the ESR spectrum was recorded after 2.5 min. The measurement conditions were as follows; central field 3,475 G, modulation width 0.2 mT, amplitude 100 mT, microwave power 1 mW, sweep width 10 mT, and temperature 298 K.

Alkyl radical scavenging assay Alkyl radicals were generated by AAPH and their scavenging effects were

investigated by the method described by Hiramoto *et al.* (18). The reaction mixture containing 20 μL of distilled water, 20 μL of the digest, 20 μL of 40 mM AAPH, and 20 μL of 40 mM POBN was incubated at 37°C for 30 min. Then, the reactants were transferred to a 50-μL glass capillary tube and fitted into the ESR spectrometer. The measurement conditions were as follows; central field 3,475 G, modulation width 0.2 mT, amplitude 500 mT, microwave power 8 mW, sweep width 10 mT, and temperature 298 K.

Hydrogen peroxide scavenging assay This assay was carried out according to the method described by Muller (19). The digest (80 μL) and 20 μL of 10 mM hydrogen peroxide were mixed with 100 μL of phosphate buffer (PBS, 0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37°C for 5 min. Thereafter, 30 μL of freshly prepared 1.25 mM ABTS and 30 μL of peroxidase (1 U/mL) were mixed and incubated at 37°C for 10 min and the absorbance was measured at 405 nm.

Metal chelating ability Metal chelating ability was determined according to the method described by Decker and Welch (20) with slight modifications. The digest (5 mL) was added to a solution of 0.1 mL of 2 mM $FeCl_2$. The reaction was started by the addition of 0.2 mL of 5 mM ferrozine solution and reaction mixture was incubated for 10 min at a room temperature under shaking condition. After incubation, the absorbance of reaction mixture was measured at 562 nm.

Lipid peroxidation inhibition by ferric thiocyanate (FTC) method The lipid peroxidation inhibitory effect of the digest was determined according to the method described by Osawa and Namiki (21) with slight modifications. Each digest (2 mg) was thoroughly mixed with 5 mL of 2.5%(w/v) linoleic acid in ethanol, 5 mL of 0.05 M phosphate buffer (pH 7.0), and 2.5 mL of distilled water. The mixture was kept in the dark at 60°C and analyzed every 24 hr interval. Fifty μL of the incubated solution was mixed with 9.7 mL of 75%(v/v) ethanol and 0.1 mL of 30%(w/v) thiocyanate. The mixture was allowed to stand for 5 min at a room temperature before adding 0.1 mL of 20 mM ferrous chloride in HCl. The absorbance was recorded at 500 nm with an enzyme-linked immunosorbant assay (ELISA) reader (Sunrise; Tecan Co., Slazburg, Austria).

Determination of the DNA damage reduction (comet assay) by CBPs The monkey kidney fibroblast cell line (Vero) was used in the comet assay. They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%(v/v) fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 unit/mL) at 37°C under a humidified atmosphere of 5% CO_2 in air.

Each AMG digest was dissolved in PBS and diluted as the final concentration of 25, 50, and 100 μg/mL. The diluted digest was then added to the vero cells as 3 different treatments. First, vero cells (4×10^4 cells/mL) were incubated without the digest (negative control) for 30 min at 37°C in a dark incubator (I). Second, vero cells (4×10^4 cells/mL) were incubated without the digest for 30 min at 37°C and damaged with 100 μM H_2O_2 (positive control) for 5 min on ice (II). Third, vero cells (4×10^4 cells/mL) were incubated with the digest of CBPs dried by HSD or FD for 30 min at 37°C in a dark incubator, and then treated with 100 μM H_2O_2 as a final concentration for 5 min on ice (III). After each treatment, digests were centrifuged at 252×g for 5 min and washed with phosphate buffered saline (PBS).

The alkaline comet assay was conducted according to the method described by Singh *et al.* (22) with slight modifications. The cell suspensions made in the previous steps were mixed with 100 μL of 0.7%(w/v) low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose. After solidification of the agarose, slides were covered with another 100 μL of 0.7%(w/v) LMA and then immersed in lysis solution (2.5 M NaCl, 500 mM EDTA, 1 M Tris, and 1%(w/v) sodium laurylasarcosine, and 1%(v/v) Triton X-100) for 90 min. Later, slides were kept in unwinding buffer for another 20 min for DNA unwinding. The slides were next placed in the electrophoresis tank containing 300 mM NaOH and 1 mM Na_2EDTA (pH 13.0), and for electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min. The slides were washed 2 times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 10 min, and treated with ethanol for another 5 min before staining with 40 μL of ethidium bromide (20 μL/mL). Measurements were made by image analysis (Komet; Andor Technology, Belfast, UK) with fluorescence microscope (Leica DMLB, Wetzlar, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of 2 replicate slides).

On-line HPLC-ABTS$^+$ radical scavenging assay A 1 g of CBPs dried by HSD and FD was extracted with 100 mL of MeOH for 24 hr at a room temperature and then filtered using micro filter 45-μM Millipore membrane. The on-line radical scavenging activity of the methanolic extracts was determined by the ABTS$^+$ assay based on the method used by Stewart *et al.* (23). In this assay, ABTS is used to produce ABTS$^+$ radicals, and the radical solution has a deep blue color. Any quenching of the ABTS$^+$ radical results in a loss of color indicated by a negative peak on the absorption profile monitored at 720 nm. A 2 mM ABTS$^+$ stock solution containing 3.5 mM potassium persulphate was prepared and kept to stand overnight at room temperature in the dark to allow radicals for stabilization. ABTS$^+$ reagent was prepared by diluting the stock solution by 8 folds in methanol. The methanolic extract (10 μL) was injected and separated using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clare, CA, USA) which equipped with binary pumps, a diode array detector (DAD), a UV/Vis detector, and an additional reagent pump. The analytical column was a reversed-phase Zorbax Eclipse XDB-C_{18}, 150 mm length, 4.6 mm i.d., and 5 μM particle size (Agilent Technologies). The mobile phase was: solvent A- acetonitrile; solvent B-water with trifluoroacetic acid (TFA) (0.1%, v/v). A gradient elution was performed with solvent A and B as follows: during 0-5, 5-15, 15-20, 20-25, 25-30, 30-50 min 10, 10-15, 10-15, 15-18, 15-18, 18-35% of solvent A, respectively. The DAD was performed in the 200-300 nm range, and the chromatographic profile was recorded at 520 nm. Sample injection volume was

Table 1. Yield, total polyphenolic, and total flavonoid contents of the different digests from citrus by-products (CBPs) dried by high speed drying (HSD) or freeze drying (FD)

	Yield (g/100 g)		Total polyphenolic (mg/100 g)		Total flavonoid (mg/100 g)	
	FD	HSD	FD	HSD	FD	HSD
AMG	43.3±0.9$^{a1)}$	41±1.4^{a}	724.4±51.2^{abc}	934.5±42.4^{d}	16.8±1.3^{c}	28.3±1.3^{a}
Celluclast	60.3±1.5^{c}	57.3±3.2^{d}	754.1±22.5^{bc}	831.8±32.6^{ab}	16.1±0.7^{bc}	25.7±1.4^{a}
Pectinase	51±3.4^{b}	47.7±2.5^{bc}	779.7±44.4^{c}	838.9±53.5^{ab}	15.6±1.1^{bc}	26±0.9^{a}
Termamyl	60.7±2.5^{c}	51.3±6.4^{c}	751.7±43.5^{bc}	823.7±35.1^{ab}	14.7±0.8^{b}	28.3±1.2^{a}
Ultraflo	60.7±1.5^{c}	58.3±0.4^{d}	657.8±31.1^{a}	854.7±40.7^{bc}	12.7±0.7^{a}	28.1±1.5^{a}
Viscozyme	55.7±3^{bc}	57.7±1.5^{d}	901.2±32.0^{d}	933.5±47.5^{d}	24.4±1.3^{d}	26±2.1^{a}
Water	43±4.3^{a}	43±3.9^{ab}	680.3±63.3^{ab}	754.8±62.9^{a}	24.4±1.2^{d}	27.9±0.6^{a}

[1)]Means with the different letters within a column were significantly different at $p<0.05$

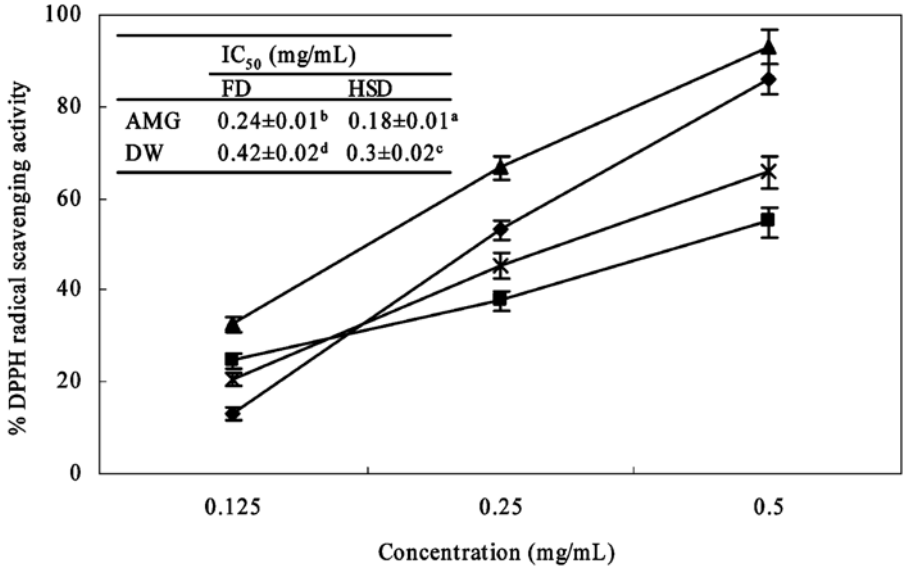

Fig. 1. DPPH radical scavenging activity of different concentrations of AMG digests and water extracts from citrus by-products (CBPs) dried by high speed drying (HSD) or freeze drying (FD). AMG digest from CBPs dried by HSD -▲-; AMG digest from CBPs dried by FD -◆-; water extract from CBPs dried by HSD -x-; water extract from CBPs dried by FD -■-.

10 μL and flow rate was 0.5 mL/min. The analysis was performed at 40°C and HPLC eluent from the detector then arrived at a 'T' piece, where the $ABTS^{+}$ was added. The $ABTS^{+}$ flow rate was 0.2 mL/min delivered by an additional Agilent 1200 pump. After mixing through a 1 mL loop maintained at 40°C, the absorbance was measured by a UV/Vis detector at 734 nm. Data were analyzed using ChemStation (Agilent Technologies).

Statistical analysis Each experiment was performed at least 3 times and results were presented as the mean±SD. Statistical comparisons of the mean values were performed by analysis of variance (ANOVA), followed by Duncans's multiple-range test using SPSS (11.5) software. Means with different letter within the table represent significant differences as $p<0.05$.

Results and Discussion

Total extractable yield, phenolic and flavonoid contents The amount of extractable compounds from the digests from CBPs dried by HSD or FD is shown in the Table 1. The enzymatic digests showed significantly higher yield content than that of water extracts which was not treated with enzymes ($p<0.05$). However, the digest from CBPs dried by HSD showed slightly lower yields than that of the digest from CBPs dried by FD. The total polyphenolic content was determined using Folin-Ciocalteu reagent and the results are shown in Table 1. It is well-known that citrus fruit and peel may contain large amount of different phytochemicals. The digests from CBPs dried by HSD exhibited significantly higher amount of polyphenolic content than that of CBPs dried by FD or water extracts from CBPs dried by HSD or FD ($p<0.05$). Flavonoid compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties; hence, the total flavonoid content was determined and was given in the Table 1. AMG digest from CBPs was used for further antioxidant assays, considering the DPPH radical scavenging activities (data not shown) of all the digests and their polyphenolic contents.

DPPH radical scavenging activity DPPH assay is known to give reliable information with regards to the antioxidant

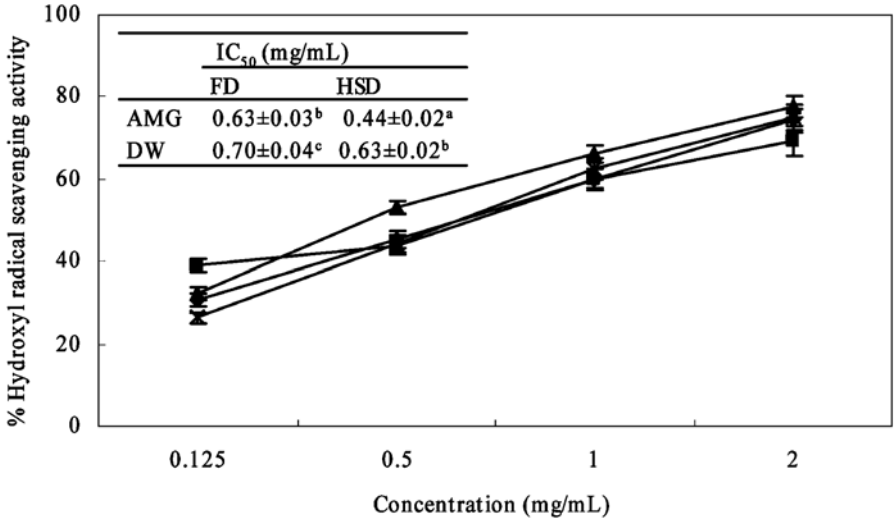

Fig. 2. Hydroxyl radical scavenging activity of different concentrations of AMG digests and water extracts from citrus by-products (CBPs) dried by high speed drying (HSD) or freeze drying (FD). AMG digest from CBPs dried by HSD-▲- ; AMG digest from CBPs dried by FD -◆-; water extract from CBPs dried by HSD -x-; water extract from CBPs dried by FD -■-.

potential of the tested compounds (24). Figure 1 demonstrated DPPH radical scavenging activity caused by different concentrations of enzymatic digests and water extracts from CBPs dried by HSD or FD. AMG digest from CBPs dried by HSD showed significantly higher DPPH radical scavenging activity (IC_{50}: 0.18±0.01 mg/mL) than that of AMG digest from CBPs dried by FD (IC_{50}: 0.24±0.01 mg/mL) or water extracts from CBPs dried by HSD or FD (IC_{50}: 0.3±0.02 and 0.42±0.02 mg/mL, respectively) ($p<0.05$). Lower IC_{50} value reflects better DPPH radical scavenging activity (25), and the AMG digest and water extract from citrus peel showed significantly lower activity than that of AMG digest or water extract from CBPs against DPPH radical scavenging (IC_{50}: 0.31±0.02 and 0.55 ±0.03 mg/mL, respectively) ($p<0.05$). The data obtained in this study revealed that the digests from CBPs dried by HSD or FD were free radical scavengers and primary antioxidants that react with DPPH radical effectively, which may be attributed to electron donating ability of the polyphenolic compounds present in the digests. Kang *et al.* (26) have reported the DPPH radical scavenging activities for citrus peel and those were lower (41.5-43.3%) than the values reported in this study.

Hydroxyl radical scavenging activity Hydroxyl radical is considered to be one of the quick initiators of lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids. Hence, the hydroxyl radical scavenging potential of the enzymatic digests and water extracts from CBPs dried by HSD or FD was evaluated and the results were depicted in the Fig. 2. The AMG digest from CBPs dried by HSD showed significantly higher activity (IC_{50}: 0.44±0.02 mg/mL) than that of the digest from CBPs dried by FD (IC_{50}: 0.63±0.03 mg/mL) or water extracts from CBPs dried by HSD or FD (IC_{50}: 0.63±0.02 and 0.70±0.04 mg/mL, respectively) ($p<0.05$). Anagnostopoulou *et al.* (27) have reported strong hydroxyl radical scavenging activity (IC_{50} ranged from 9.7 to 275 mg/dry extracts/mL) for the different extracts, fractions and residues of citrus peel using luminol enhanced chemiluminescence method. Also, Yi *et al.* (28) have reported the hydroxyl radical scavenging activity of citrus and hesperidin (IC_{50} of extracts from citrus, hesperidin, nobiletin and tangeretin was 76.22, 35.26, 96.97, and 103.20 mg/mL, respectively) and indicated that those activities were due to the presence of multi-hydroxyl groups of the phenolic ring in the hesperidin molecule.

Alkyl radical scavenging activity An alkyl radical spin adduct was observed (a^N=15.5 G, a^H=2.7 G) when AAPH was incubated with the spin trap 4-POBN at 37°C for 30 min. ESR signals were decreased with the addition of the tested compounds (spectrum not shown). The enzymatic digests from CBPs dried by HSD or FD showed strong activities against alkyl radical formation and it was dose-dependent (Fig. 3). The AMG digests from CBPs dried by HSD or FD showed the equal IC_{50} values (IC_{50}: 0.08±0.003 mg/mL) for alkyl radical scavenging. However, those values were higher than those of their water counterparts.

Hydrogen peroxide scavenging activity Hydrogen peroxide is not very reactive, but it can sometimes be toxic to cells, since hydrogen peroxide is capable of producing hydroxyl radicals inside the cell (29). Hence, removing of the excess hydrogen peroxide from the system is needed to protect the biomolecules from damage. AMG digests were capable of scavenging hydrogen peroxide in a dose-dependent manner and the results were shown in Fig. 4. AMG digest from CBPs dried by HSD showed significantly higher activity (IC_{50}: 0.57±0.02 mg/mL) in hydrogen peroxide scavenging than that of AMG digest from CBPs dried by FD (IC_{50}: 0.66±0.03 mg/mL), as well as water extracts from CBPs dried by HSD or FD (IC_{50}: 0.95±0.03 and 1.0±0.04 mg/mL, respectively) ($p<0.05$). Yi *et al.* (28) have reported the hydrogen peroxide scavenging activity of citrus and flavonoids (IC_{50} of extracts from citrus, hesperidin, nobiletin, and tangeretin was 11.8, 16.1, 19.2, and 17.5 mg/mL, respectively) and indicated strong activity.

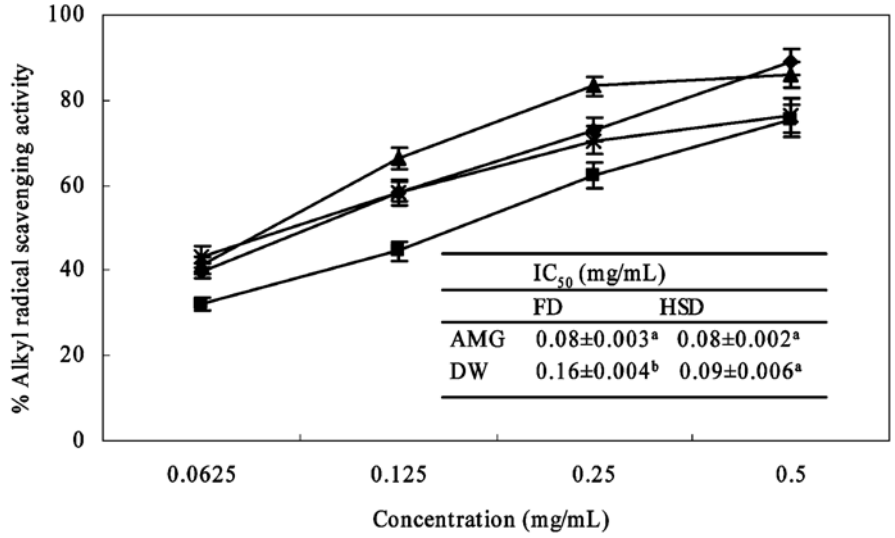

Fig. 3. Alkyl radical scavenging activity of different concentrations of AMG digests and water extracts from citrus by-products (CBPs) dried by high speed drying (HSD) or freeze drying (FD). AMG digest from CBPs dried by HSD -▲-; AMG digest from CBPs dried by FD -◆-; water extract from CBPs dried by HSD -x-; water extract from CBPs dried by FD -■-.

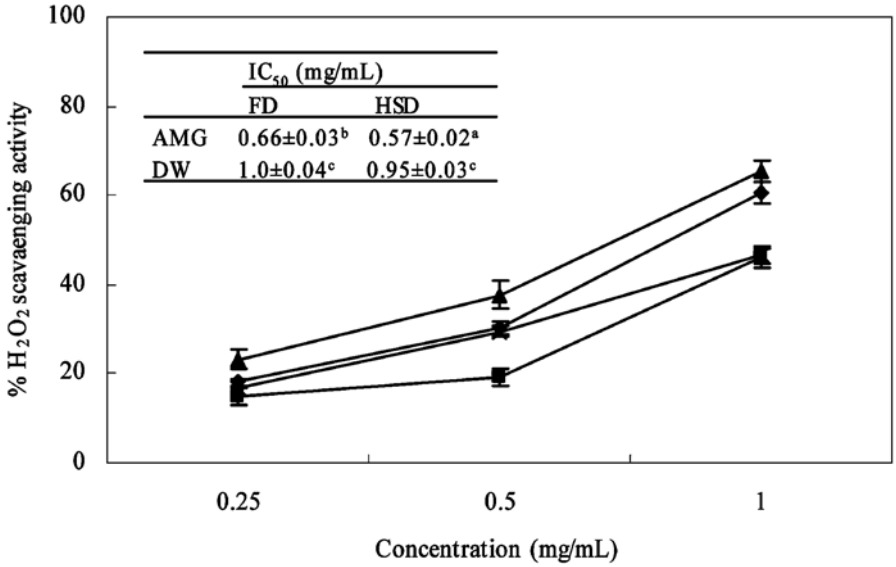

Fig. 4. Hydrogen peroxide scavenging activity of different concentrations of AMG digests and water extracts from citrus by-products (CBPs) dried by high speed drying (HSD) or freeze drying (FD). AMG digest from CBPs dried by HSD -▲-; AMG digest from CBPs dried by FD -◆-; water extract from CBPs dried by HSD -x-; water extract from CBPs dried by FD -■-.

Metal chelating activity It is well-known that transition metal ions are involved in many oxidation reactions. Hence, the measurement of chelating ability is important for evaluating free radical scavenging activity of a compound (30). In the present study, the metal chelating activities were determined by measurement of the reduction rate of red color, which was quantitatively formed by ferrozine reacting with ferrous ions. Figure 5 showed the chelating activities of the enzymatic digests from CBPs dried by HSD or FD on ferrous ions. The metal chelating activity of AMG digest from CBPs dried by HSD (IC_{50}: 0.49±0.02) was significantly higher than that of its counterpart AMG digest from CBPs dried by FD (IC_{50}: 1.5±0.05) as well as water extracts from CBPs dried by HSD or FD (IC_{50}: 3.1±0.12 and 3.2±0.12, respectively) ($p<0.05$).

Lipid peroxidation inhibitory activity The ferric thiocyanate method was employed to determine the amount of peroxide at the initial stage of lipid peroxidation, and it consists of a series of free radical-mediated chain reactions. When different digests were added, a significant inhibition of lipid peroxidation was observed (Fig. 6). The autooxidation of linoleic acid (control: without adding digests) was accompanied by a rapid increase of peroxide value. Each digests showed strong antioxidant activities in inhibition of linoleic acid peroxidation as compared with the control. However, the inhibitory activities of CBPs dried by HSD or FD were lower than that of BHT but higher than that of α-tocopherol. In FTC method, hydroperoxides generated from linoleic acid which autoxidized during the incubation period were indirectly measured. Ferrous

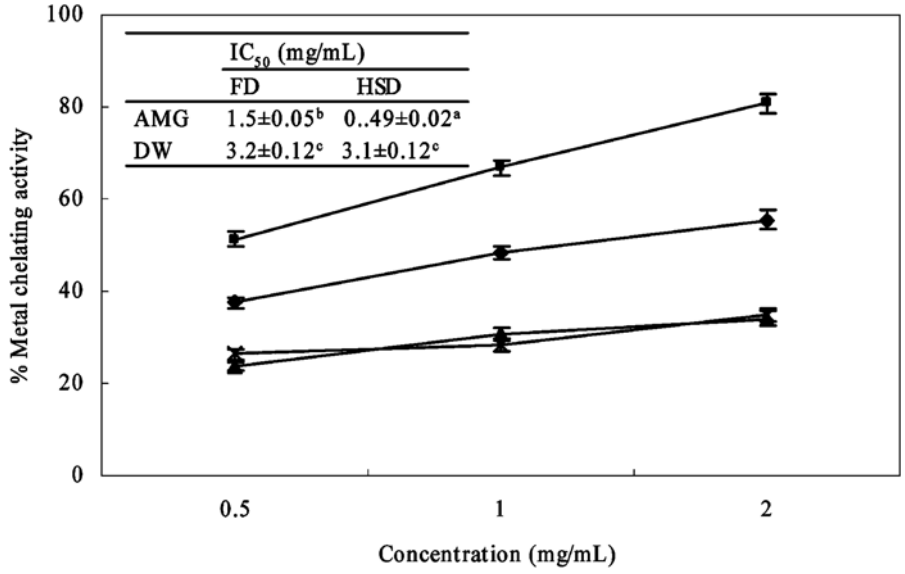

Fig. 5. Metal chelating activity of different concentrations of AMG digests and water extracts from citrus by-products (CBPs) dried by high speed drying (HSD) or freeze drying (FD). AMG digest from CBPs dried by HSD -▲-; AMG digest from CBPs dried by FD -◆-; water extract from CBPs dried by HSD -**x**-; water extract from CBPs dried by FD -■-.

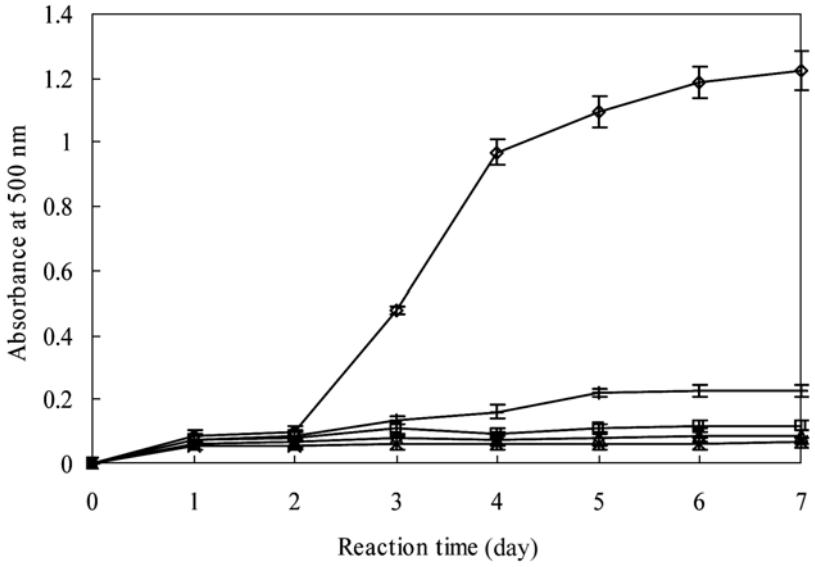

Fig. 6. Lipid peroxidation inhibition of AMG digests from citrus by-products (CBPs) dried by high speed drying (HSD) or freeze drying (FD). Control -◇-; AMG digest from CBPs dried by HSD -△-; AMG digest from CBPs dried by FD -□-; BHT -**x**-; α-tocopherol -+-. The concentration of the AMG digest, BHT and α-tocopherol was 2 mg/mL.

chloride and thiocyanate react with each other to produce ferrous thiocyanate by means of hydroperoxides (31). Kang *et al.* (26) and Rehman (32) have also reported the lipid peroxidation inhibitory activities of citrus peel using different model systems. Further, Manthey (33) has reported the antioxidant property observed in orange peel ultra filtered molasses due to the presence of polyphenolic compounds such as flavanones, flavone glycosides, polymethoxylated flavone, hydroxyl cinnamates, and other various phenolic glycosides. The strong lipid peroxidation inhibitory activities showed by AMG digests from CBPs dried by HSD or FD could be attributed to the presence of various polyphenolics including flavonoid compounds.

Effect of the extracts from CBPs on cell damage DNA is the genetic material which controls the cellular functions and can be damaged as a result of several factors such as reactive oxygen species, smoke, heat, toxic chemicals, and ultraviolet light. The sequence of the DNA base pairs can be changed and leads errors/disorders in replicating DNA, if the damage could not be repaired by the existing DNA repair mechanisms. The DNA damage of cultured vero cells was artificially induced by H_2O_2, and the ability of the enzymatic digests from CBPs dried by HSD or FD to inhibit the damage was evaluated by the fluorescence intensity in the tail using comet assay. DNA damage is well known sensitive biological markers for evaluating oxidative

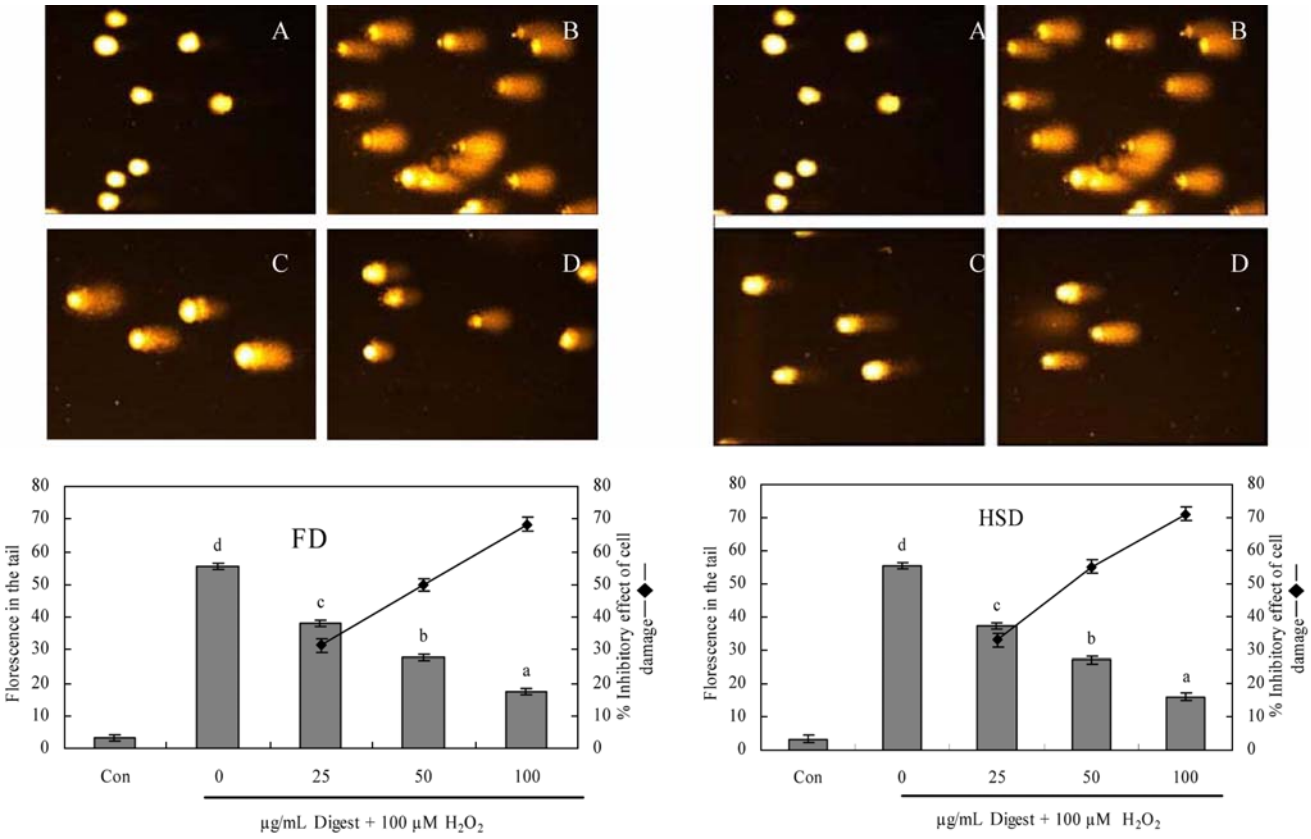

Fig. 7. Effect of AMG digest from citrus by-products (CBPs) dried by high speed drying (HSD) or freeze drying (FD) on H_2O_2-induced DNA damage in vero cells and their comet images. (A) negative control (untreated vero cells), (B) positive control (vero cells treated with 100 µM H_2O_2) (C) vero cells treated with 50 µg/mL AMG digest+100 µM H_2O_2, (D) vero cells treated with 100 µg/mL AMG digest+100 µM H_2O_2.

stress caused by ROS (34). As shown in Fig. 7 the inhibition of DNA damage by the enzymatic digests from CBPs dried by HSD or FD was investigated with 3 different concentrations. With the increased concentrations of the digests, increased inhibitory effect against H_2O_2-induced cell damage was observed. The enzymatic digests from CBPs dried by HSD or FD showed almost similar inhibitory effects (about 70%) at the concentration of 100 µg/mL. In the group treated with only hydrogen peroxide, the DNA was completely damaged, but the addition of the enzymatic digests with hydrogen peroxide reduced the damage indicating that the AMG digests have the ability to inhibit the DNA damage induced by hydrogen peroxide.

On-line HPLC analysis of bioactive compounds and antioxidant activity The described on-line HPLC-$ABTS^+$ method can be used for a rapid assessment of pure antioxidants and antioxidative activities in complex mixtures (23,35,36). This method was applied to analyze the antioxidant ability of each component from CBPs dried by HSD comparing with that of CBPs dried by FD. Simultaneously obtained UV (positive) and $ABTS^+$ (negative) radical quenching chromatograms using gradient elution of the crude extracts from CBPs dried by HSD or FD isolated with acitonitrile and water with TFA are presented in Fig. 8. HPLC elute was mixed with a stabilized solution of $ABTS^+$ radical followed by HPLC separation and the separated fractions were directed to a UV/Vis detector monitoring absorbance at 734 nm. The absorbance of the $ABTS^+$ radical reaction mixture was decreased due to the reduction by antioxidant compounds in the extract from CBPs dried by HSD and FD and was indicated by the negative peak. $ABTS^+$-based antioxidant activity profile showed that several compounds contribute for the activity. Hesperidin was found as a major compound in both extracts from which remarkable antioxidant abilities was described. It has long been recognized that orange peel represents a promising source of hesperidin (37,38) while it is an effective antioxidant, since it is able to quench the oxygen free radicals which involved in cancer (39,40). Other than hesperidin, the extracts contain several other flavonoids in minor amounts (neohesperidin, sinesitin, and tetramethoxyflavone, etc). Hence, noticeable amount of hesperidin, minor flavonoid, and other polyphenolic compounds in the extracts from CBPs dried by HSD may be attributed to the observed antioxidant activities in this study.

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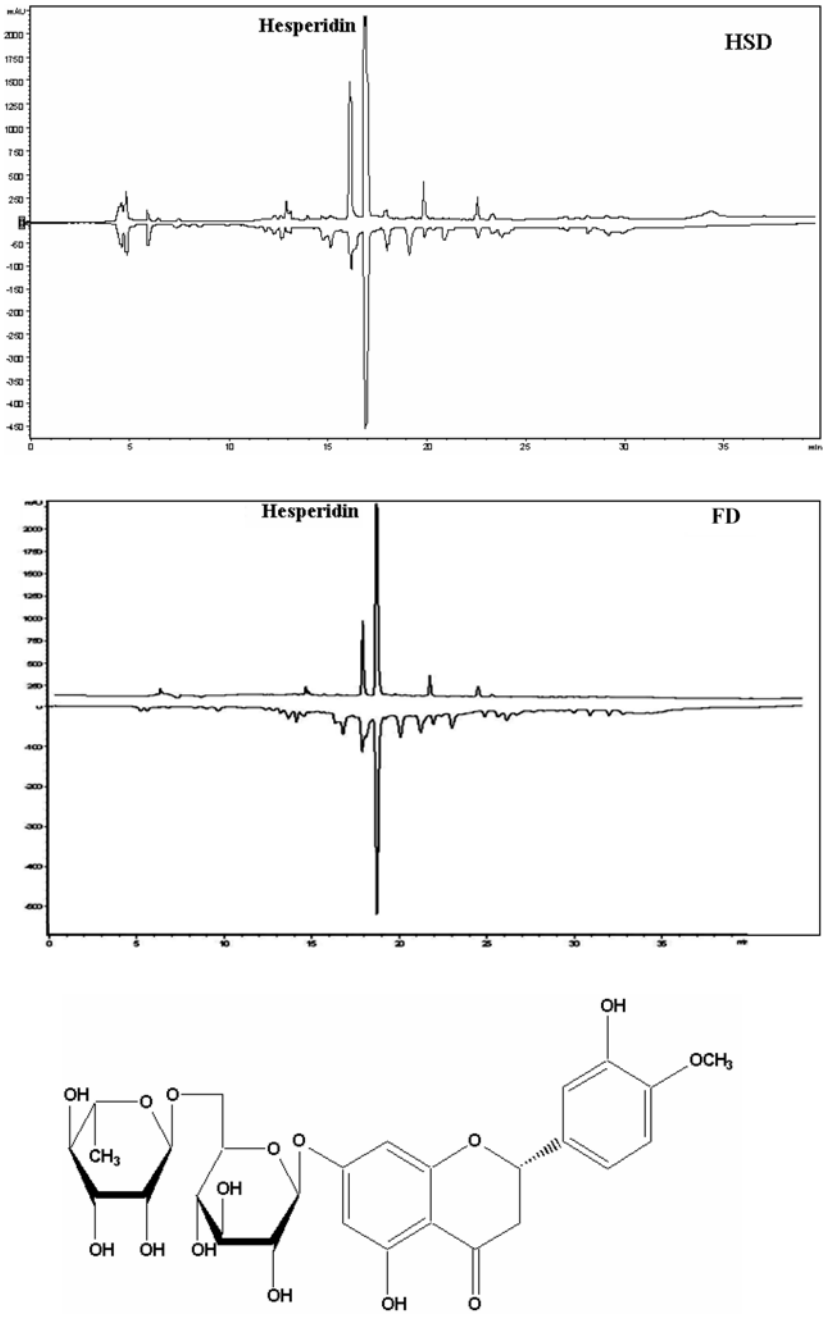

Fig. 8. Separation of antioxidants by HPLC coupled with on-line ABTS$^+$ assay. The upper trace shows the peaks of antioxidant compounds detected at 280 nm following injection of 10 μL of crude methanolic extracts, whilst the lower trace shows their antioxidant activities against ABTS$^+$ detected at 734 nm.

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