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Antioxidative and Neuroprotective Effects of Enzymatic Extracts from Leaves of *Perilla frutescens* var. *japonica*

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Abstract The antioxidative activity of various enzymatic extracts from leaves of *Perilla frutescens* var. *japonica* was evaluated by measuring 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and alkyl radical scavenging activity using an electron spin resonance (ESR) spectrometer. For this study, the leaves were enzymatically hydrolyzed by 8 carbohydrases (Dextrozyme, AMG, Promozyme, Maltogenase, Termamyl, Viscozyme, Celluclast, and BAN) and 9 proteases [Flavourzyme, Neutrase, Protamex, Alcalase, PP-trypsin (trypsin from porcine pancreas), papain, pepsin, α-chymotrypsin, and BP-trypsin (trypsin from bovine pancreas)]. The DPPH radical scavenging activities of Promozyme and Alcalase extracts were the highest, and the IC_{50} values were 77.25 and 109.66 μg/mL, respectively. All enzymatic extracts of the leaves scavenged hydroxyl radical, and the IC_{50} values of Celluclast and pepsin extracts which were the highest activity were 243.34 and 241.86 μg/mL, respectively. The BAN and α-chymotrypsin extracts showed the highest scavenging activities, and the IC_{50} values were 21.13 and 33.23 μg/mL, respectively. The pepsin extracts from the leaves showed protective effect on H_2O_2-induced DNA damage. In addition, the pepsin extracts decreased cell death in PC-12 cells against H_2O_2-induced oxidative damage. The findings of the present study suggest that enzymatic extracts of the leaves possess antioxidative activity.

Keywords: *Perilla frutescens* var. *japonica*, DNA damage, free radical, enzymatic extracts, electron spin resonance (ESR) spectrometer

Introduction

The etiology of a range of diseases is associated with the generation of excess reactive oxygen species (ROS), and ROS generation may also be associated with external stimuli. Ultra violet (UV) and high energy irradiation, the metabolism of some xenobiotics, air pollutants, the redox cycling of quinines, and nitroaromatics are all associated with ROS generation (1).

A shift in the balance between ROS generation and destruction to overproduction or decreased detoxication is associated with many diseases such as cancer, gastric ulcers, Alzheimer's, arthritis, renal injury, and ischemic reperfusion (3-11). Therefore, there is a continuing search for better and more effective antioxidants. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. The synthetic antioxidants such as butylated hydroxyanisol (BHA), butylated hydroxyltoluene (BHT), *tert*-butylhydroquinone (TBHQ), and propyl gallate (PG) are added in food during processing to suppress lipid peroxidation and consequently to improve food quality and stability. However the use of these antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (2). Therefore, much higher attention of investigators and consumers are focusing on natural antioxidant for use in foods or medicinal materials to replace synthetic antioxidant.

Perilla frutescens var. *japonica* has been widely used as a source for folk medicine and/or foods, particularly in southeast Asia (12). In addition, it has been known for several biological activities such as antioxidative effect (13), anti-allergic effect (14,15), anti-tumor effects (16,17), and anti-inflammatory effects (16-19). However, research into the antioxidative effect of enzymatic extracts from the leaves of *P. frutescens* has not been evaluated until now. In addition, our earlier study showed that free radical scavenging activities of enzymatic extracts from the leaves were higher than methanol extracts from the leaves. As part of our ongoing investigation of bioactive substances from higher herbal plant by enzymatic hydrolysis, we have focused on our attention on the production of antioxidant substances such as peptides (20-24) and/or carbohydrates (25-27).

In the present study, the leaves of *P. frutescens* were enzymatically hydrolyzed to prepare water-soluble extracts from various potential antioxidants such as proteins, carbohydrates, and polyphenols. In addition, the radical scavenging activity of the enzymatic extracts was investigated on DPPH, hydroxyl, and alkyl radicals by using an electron spin resonance (ESR) spectrometer. The neuroprotective effect and DNA protective effect against H_2O_2-induced oxidative stress of enzymatic extracts from the leaves were investigated.

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Materials and Methods

Materials 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), (4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), and 4 proteases such as papain, pepsin, α-chymotrypsin, BP-trypsin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and pBR 322 DNA was purchased from Fermentas (Hanover, MD, USA). Eight carbohydrases such as Dextrozyme, AMG, Promozyme, Maltogenase, Termamyl, Viscozyme, Celluclast, and BAN, and 5 proteases including Flavourzyme, Neutrase, Protamex, Alcalase, and PP-trypsin were donated from Novozymes (Bagsvaerd, Denmark). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Invitrogen Corporation (Carlsbad, CA, USA). RNase A and Tween-20 were supplied by Novagen (Darmstadt, Germany) and USB (Cleveland, OH, USA), respectively. In addition, the *P. frutescens* was sourced from a local market (Chungju, Korea). All other reagents were of the highest grade available commercially. IC_{50} represents the concentration of an inhibition that is required for 50% inhibition of each radical generation.

Preparation of enzymatic extracts from leaves of *P. frutescens* The leaves were enzymatically hydrolyzed with 8 carbohydrases and 9 proteases to prepare water soluble extracts, useful oligosaccharides, and peptides (28,29). The samples were pulverized into powder using a grinder. The optimum pHs, temperatures, and characterizations of various enzymes were summarized in Table 1 and the enzymatic extracts were obtained according to the method described by Park *et al.* (21). Briefly, 100 mL of buffer solution was added to 2 g of the dried sample, and then 40 μL (or mg) of each enzyme was added after pre-incubation for 30 min. The enzymatic hydrolysis reactions were performed for 8 hr to achieve an optimum hydrolytic level and immediately heated at 100°C for 10 min. Finally, the enzymatic extracts were obtained after filtering the supernatant, lyophilizing, and then stored at −20°C until use.

Determination of protein and carbohydrate contents of enzymatic extracts The protein contents of various enzymatic extracts was determined according to the method of Lowry *et al.* (30) using bovine serum albumin as a standard. In addition, the chemical analysis for the determination of total carbohydrate was adapted from the phenol-sulphuric method as described by Dubois *et al.* (31).

DPPH radical scavenging activity DPPH radical scavenging activity was measured using the method described by Nanjo *et al.* (32). A sample solution of 60 μL of each enzymatic extracts, was added to 60 μL of DPPH (60 μM) in methanol solution. After mixing vigorously for 10 sec, the solution was then transferred into a 100 μL Teflon capillary tube, and the scavenging activity of each enzymatic extract on DPPH radical was measured using a JES-FA ESR spectrometer (Jeol Ltd., Tokyo, Japan). A spin adduct was measured on an ESR spectrometer exactly 2 min later. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5, and temperature, 298 K.

Hydroxyl radical scavenging activity Hydroxyl radicals were generated by iron-catalyzed Haber-Weiss reaction (Fenton-driven Haber-Weiss reaction), and the generated

Table 1. Optimum hydrolyzation conditions of particular enzymes

Enzyme	Optimum conditions		Buffer used[1)]	Enzyme composition
	pH	Temperature (°C)		
Protamex	7.0	50	0.1 M PB	Hydrolysis of food proteins
Flavourzyme	7.0	50	0.1 M PB	Containing both endoprotease and exopeptidase activities
Neutrase	7.0	50	0.1 M PB	Endoprotease
Pancreatic trypsin NOVO	7.0	37	0.1 M PB	Serine protease (from porcine pancreas)
Alcalase	7.0	50	0.1 M PB	Endo protease
Papain	7.0	37	0.1 M PB	Cysteine protease (from papaya latex)
Pepsin	2.0	37	0.2 M GH	Cleavage C-terminal to Phe, Leu, and Glu
α-Cymotrypsin	8.0	37	0.1 M PB	Serine protease (from bovine pancreas)
Trypsin	8.0	37	0.1 M PB	Serine protease (from bovine pancreas)
Promozyme 400L	5.0	60	0.1 M SB	Debranching enzymes known as pullulanases
Celluclast 1.5LFG	4.5	50	0.1 M SB	Catalyzing the breakdown of cellulose into glucose, cellobiose, and higher glucose polymer
Maltogenase L	5.0	60	0.1 M SB	α-Amylase
BAN 480L	7.0	70	0.1 M PB	Hydrolyzing 1,4-α-glucosidic linkages in amylose and amylopectin
Viscozyme L	4.5	50	0.1 M SB	Arabanase, celluiase, β-glucanase, hemi-cellulase, and xyianase
Termamyl SC	6.0	60	0.1 M PB	Heat-stable α-amylase
Dextrozyme E	4.5	60	0.1 M SB	Glucoamylase and pullulanase
AMG 300L	4.5	60	0.1 M SB	Exo-1,4-α-D-glucosidase

[1)]In enzymatic hydrolysis: PB, phosphate buffer; GH, glycin-HCl buffer; SB, sodium acetate-acetic acid buffer.

hydroxyl radicals rapidly reacted with nitrone spin-trap DMPO (33). The resultant DMPO-OH adduct was detectable with an ESR spectrometer. Briefly, 0.2 mL of each enzymatic extracts with various concentrations was mixed with 0.2 mL of DMPO (0.3 M), 0.2 mL of $FeSO_4$ (10 mM), and 0.2 mL of H_2O_2 (10 mM) in a phosphate buffer solution (pH 7.2), and then transferred into a 100 μL Teflon capillary tube. After 2.5 min, ESR spectrum was recorded using an ESR spectrometer. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5, and temperature, 298 K.

Alkyl radical scavenging activity Alkyl radicals were generated by AAPH. The phosphate-buffered saline (PBS, pH 7.4) reaction mixtures containing 10 mM AAPH, 10 mM 4-POBN, and indicated concentrations of tested samples were incubated at 37°C in a water bath for 30 min and then transferred to a 100 μL teflon capillary tube. The spin adduct was recorded on an ESR spectrometer. Measurement conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5, and temperature, 298 K.

Protective effect of the enzymatic extracts against H_2O_2-induced DNA damage To investigate the protection effects of the enzymatic extracts on H_2O_2-induced DNA damage, the reaction was conducted in an eppendorf tube at a total volume of 13 μL containing 0.5 μg of pBR 322 DNA in 3 μL of 50 mM phosphate buffer (pH 7.4), 3 μL of 0.02 mM $FeSO_4$, and 2 μL of the enzymatic extracts at various concentrations. Then 4 μL of 30% H_2O_2 was added, and the mixture was incubated at 37°C for 1 hr (34). The mixture was subjected to 0.8% agarose gel electrophoresis, and DNA bands (supercoiled, linear, and open circular) were stained with ethidium bromide.

Cell culture PC-12 cells used in this study were obtained from the American Type Culture Collection, and were used for no more than 10-12 passages. Growth medium consisted of DMEM supplemented with 10% heat-inactivated FBS and antibiotics (10 μg/mL penicillin-streptomycin). The cells were incubated in a humidified incubator at 37°C with 5% CO_2 and 95% air. All treatments were performed at 30% confluence of cells.

Flow cytometer For sub-G1 and cell cycle analysis, PC-12 cells were suspended in ethanol with 0.5% Tween-20 and left for 24 hr at 4°C. The cells were harvested by centrifugation and resuspended in 1.0 mL of PBS with 0.05 mg/mL of propidium iodide and 10 μg/mL of RNase A, and incubated at 37°C for 30 min. The analysis of apoptotic cell death was performed by measuring the hypodiploid DNA contents using a flow cytometer (FACS-caliber; Becton Dickinson, Franklin Lakes, NJ, USA). The cells in sub-G1 population was considered as apoptotic cells and percentage of each phase of cell cycle was determined.

Statistical analysis The data are presented as mean±SD. The values were evaluated by one-way analysis of variance (ANOVA) followed by post-hoc Duncan's multiple range tests. The paired *t*-test was used for comparisons between DNA damage and the leaves-treated group. All analyses were performed using an SAS system (SAS Institute, Cary, NC, USA).

Results and Discussion

Yield of enzymatic extracts from the leaves The yield of enzymatic extracts from the leaves by the 8 carbohydrases such as Dextrozyme, AMG, Promozyme, Maltogenase, Termamyl, Viscozyme, Celluclast, and BAN were 46.7, 48.8, 56.0, 56.2, 48.3, 57.9, 59.0, and 80.3%, respectively (data not shown). In addition, the yield of enzymatic extracts by the 9 proteases such as Flavourzyme, Neutrase, Protamex, Alcalase, PP-trypsin, papain, pepsin, α-chymotrypsin, and BP-trypsin were 93.9, 77.5, 89.4, 93.6, 70.3, 74.8, 60.9. 88.8, and 78.9%, respectively (data not shown).

The yield of all enzymatic extracts was higher than that of methanolic extracts. The yield of methanolic extracts from the leaves was 23.1% (data not shown). Therefore, these results indicate that the efficiency of enzymatic extracts from the leaves is better than the methanolic extracts for industrial application.

Total protein and carbohydrate contents of enzymatic extracts from the leaves The leaves were enzymatically hydrolyzed to prepare water soluble extracts assisted by various carbohydrases and proteases. The total protein content of hydrolytic extracts from the leaves by the 8 carbohydrases such as Dextrozyme, AMG, Promozyme, Maltogenase, Termamyl, Viscozyme, Celluclast, and BAN were 133.00, 147.29, 95.14, 46.21, 210.5, 140.86, 126.93, and 116.21 mg/g, and the protein contents of the enzymatic extracts by proteases such as Flavourzyme, Neutrase, Protamex, Alcalase, PP-trypsin, papain, pepsin, α-chymotrypsin, and BP-trypsin were 122.29, 141.57, 66.57, 145.86, 88.71, 75.50, 144.43, 83.36, and 138.00 mg/g, respectively (data not shown). The total protein contents of AMG and Alcalase extracts among various carbohydrases and proteases extracts from the leaves were the highest and the values were 147.29 and 145.86 mg/g, respectively (data not shown).

The carbohydrate contents of hydrolytic extracts from the leaves by the carbohydrases such as Dextrozyme, AMG, Promozyme, Maltogenase, Termamyl, Viscozyme, Celluclast, and BAN were 32.69, 66.58, 42.34, 77.25, 105.09, 37.37, 68.29, and 29.72 mg/g, and the carbohydrate contents of the enzymatic extracts prepared by proteases such as Flavourzyme, Neutrase, Protamex, Alcalase, PP-trypsin, papain, pepsin, α-chymotrypsin, and BP-trypsin were 20.60, 24.75, 13.83, 20.57, 48.70, 14.91, 62.22, 42.78, and 37.47 mg/g, respectively (data not shown). In addition, Termamyl and pepsin extracts among various carbohydrases and proteases extracts from the leaves showed the highest carbohydrate contents, and the values were 105.09 and 62.22 mg/g, respectively (data not shown).

DPPH radical scavenging activity DPPH radical is stable free radical as a free radical donor, which has been used to evaluate free radical scavenging activity of natural antioxidants. In this study, the DPPH radical scavenging

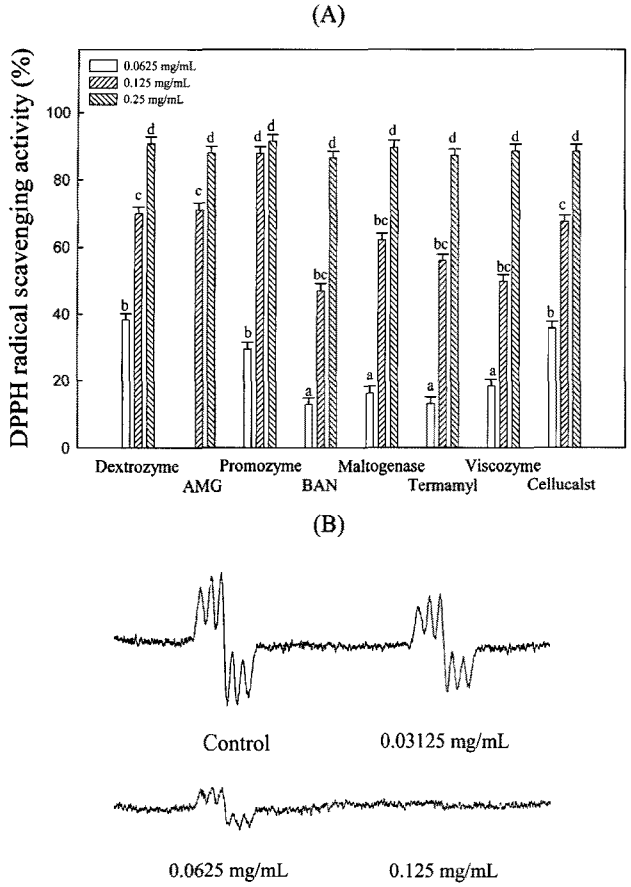

Fig. 1. DPPH radical scavenging activity of various enzymatic extracts by carbohydratic hydrolysis from leaves of *P. frutescens* (A) and ESR spectra of Promozyme extracts from leaves of *P. frutescens* (B). Means±SD of determinations were made in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

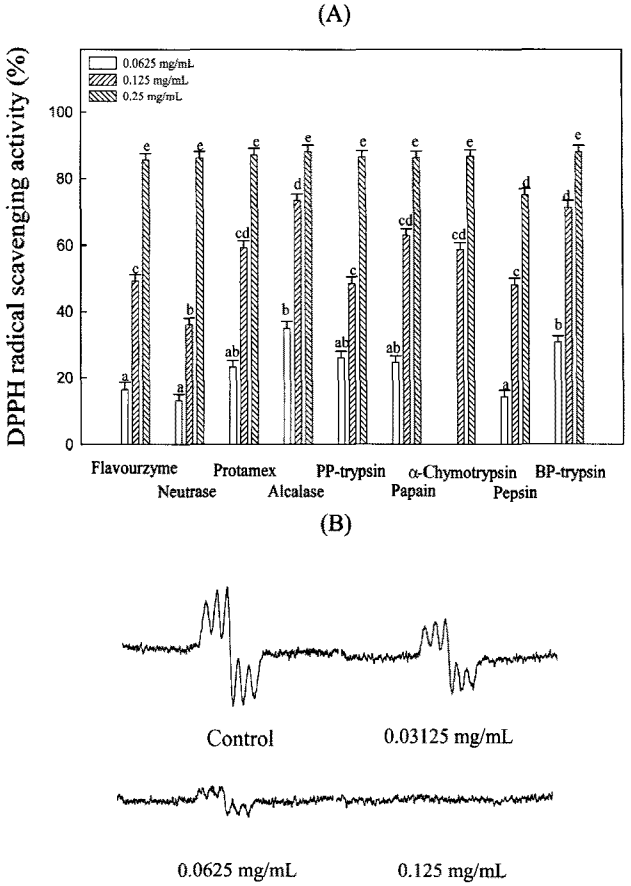

Fig. 2. DPPH radical scavenging activity of various enzymatic extracts by proteolytic hydrolysis from leaves of *P. frutescens* (A) and ESR spectra of Alcalase extracts from leaves of *P. frutescens* (B). Means±SD of determinations were made in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

activity of various carbohydrases and proteases extracts are shown in Fig. 1 and 2. It was observed that carbohydratic extrats by Promozyme, Dextrozyme, Maltogenase, Viscozyme, Celluclast, Termamyl, and BAN had scavenging activity 91.47, 90.78, 89.62, 88.60, 88.45, 87.25, and 86.57% at 0.25 mg/mL on DPPH radical, and the scavenging activity of AMG was 87.96% at 0.125 mg/mL. The proteolytic extracts by Alcalase, BP-trypsin, Protamex, α-chymotrypsin, PP-trypsin, papain, Neutrase, pepsin, and Flavourzyme from the leaves scavenged 88.18, 88.07, 87.17, 86.76, 86.46, 86.27, 86.16, 85.59, and 75.09% at 0.25 mg/mL on DPPH radical. The radical scavenging activity was concentration-dependent manners. However there was no DPPH radical scavenging activity of methanol extracts from the leaves (data not shown). In addition, Promozyme and Alcalase extracts exhibited the strongest scavenging activities among 8 carbohydrases and 9 proteases extracts, and the IC_{50} values were 77.25 and 109.66 μg/mL, respectively. These activities are higher than earlier results of the aqueous extracts from the leaves. The IC_{50} value of the aqueous extracts was 407 μg/mL and the IC_{50} value of ascorbic acid was 35.5 μg/mL as a reference (13). However, the earlier results were measured using colorimetric method, therefore it is impossible to compare with our results. Meanwhile, Calliste *et al.* (35) reported that DPPH radical scavenging activity of another plant, sweet chestnut, was 71 μg/mL, and the activity of vitamin E was 25 μg/mL as a reference. Although DPPH radical scavenging activity of the leaves was lower than vitamin E, these results indicate that enzymatic extracts from the leaves appear to be good potential candidates for DPPH radical scavenger.

Hydroxyl radical scavenging activity Hydroxyl radicals generated in the Fe^{2+}/H_2O_2 system were trapped by DMPO, forming a spin adduct detected by an ESR spectrometer, and the typical 1 : 2 : 2 : 1 ESR signal of the DMPO-OH adduct was observed as shown in Fig. 3 and 4. They may be due to the paramagnetic impurities contained in unpurified commercial DMPO (33). The height of the third peak of the spectrum represents the relative amount of DMPO-OH adduct. As shown in Fig. 3, it was observed that the hydroxyl radical scavenging activities of Celluclast, Promozyme, Dextrozyme, Maltogenase, Viscozyme, AMG, Termamyl, and BAN from the leaves were 78.08, 77.09, 68.55, 66.39, 66.02, 63.64, 63.09, and 44.80% at 1 mg/mL, respectively. As shown in Fig. 4, it was observed that 1 mg/mL of the extracts prepared with 9 types of proteases such as pepsin, Alcalase, papain, PP-trypsin, Protamex, α-

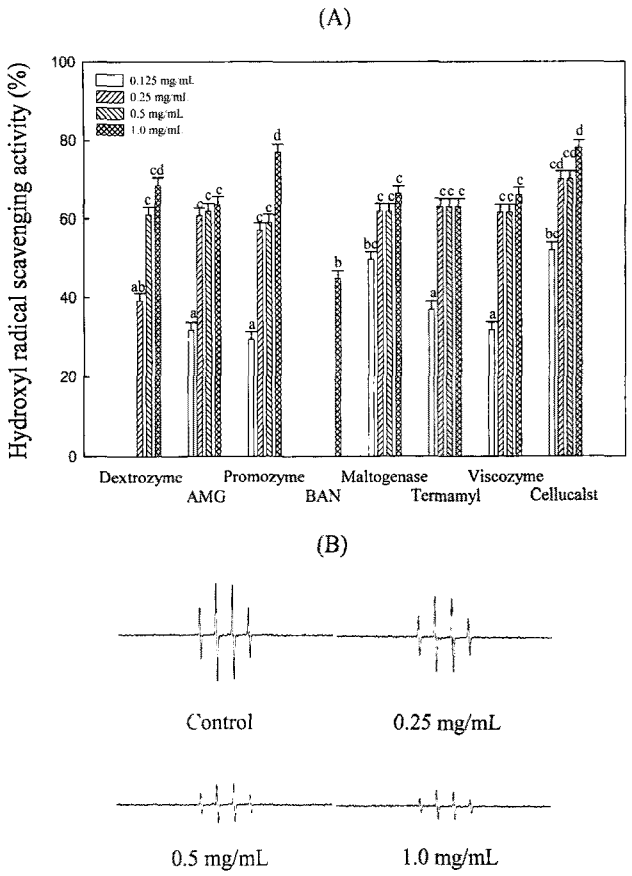

Fig. 3. Hydroxyl radical scavenging activity of various enzymatic extracts by carbohydratic hydrolysis from leaves of *P. frutescens* (A) and ESR spectra of Celluclast extracts from leaves of *P. frutescens* (B). Means±SD of determinations were made in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

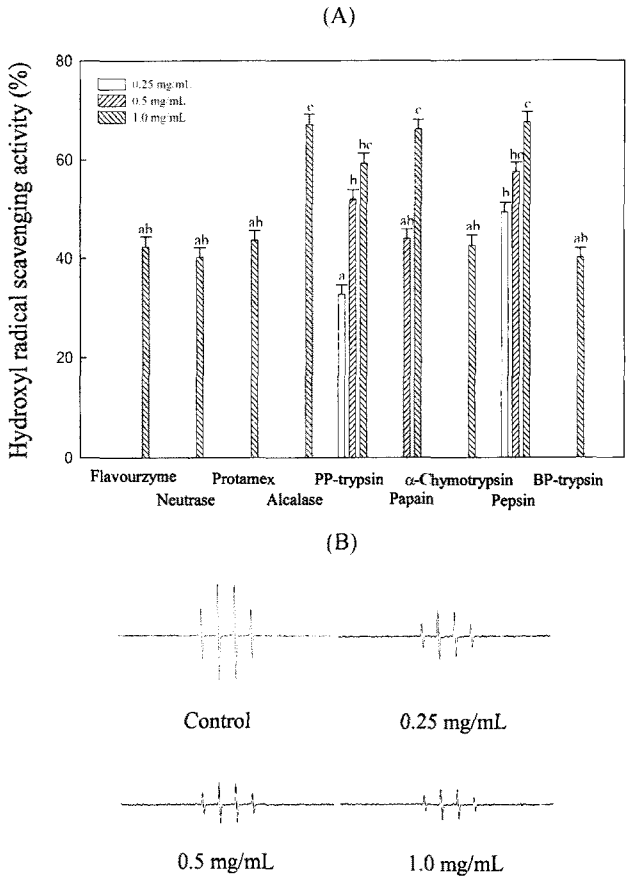

Fig. 4. Hydroxyl radical scavenging activity of various enzymatic extracts by proteolytic hydrolysis from leaves of *P. frutescens* (A) and ESR spectra of pepsin extracts from leaves of *P. frutescens* (B). Means±SD of determinations were made in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

chymotrypsin, Flavourzyme, Neutrase, and BP-trypsin also scavenged 67.58, 67.13, 66.07, 59.28, 43.56, 42.60, 42.32, 40.12, and 40.03% on hydroxyl radical, respectively. The decrease of the amount of DMPO-OH adduct was expressed by ESR signals (Fig. 3B, 4B) and the radical scavenging activity was concentration-dependent manners. Meanwhile, hydroxyl radical scavenging activity of methanol extracts from the leaves was 61.70% at 1 mg/mL which value was not higher compared to enzymatic extracts. In addition, the Celluclast and pepsin extracts were the highest scavenging activities among various carbohydrases and proteases extracts, and the IC_{50} values were 243.34 and 241.86 μg/mL, respectively. These scavenging activity were similar (methanol extracts) or higher (aqueous extracts) than the scavenging activity of another plant using ESR spectrometer (35). Theses results indicate that antioxidant property of the leaves.

Alkyl radical scavenging activity The alkyl radical spin adduct of 4-POBN/free radicals was generated from AAPH at 37°C for 30 min, and the decrease of ESR signals was observed with the dose increment of all enzymatic extracts (Fig. 5, 6). The extracts from the leaves exhibited the alkyl radical scavenging activities, and the scavenging activities of BAN, Viscozyme, Celluclast, Dextrozyme, AMG, Maltogenase, Promozyme, and Termamyl were 82.52, 82.35, 82.19, 82.08, 81.97, 81.45, 01.06, and 79.69% at 1 mg/mL (Fig. 5). In addition, 1 mg/mL of the extracts hydrolyzed from the leaves by the 9 types of proteases such as α-chymotrypsin, BP-trypsin, Flavourzyme, Neutrase, Protamex, pepsin, Alcalase, PP-trypsin, and papain also scavenged 87.98, 84.98, 80.45, 80.24, 76.22, 74.48, 74.11, 74.00, and 60.88% at 1 mg/mL (Fig. 6). Meanwhile, alkyl radical scavenging activity of methanol extracts from the leaves was 66.32% at 0.0625 mg/mL which value was higher than alkyl radical scavenging activity of enzymatic extracts (data not shown). Additionally, BAN, and α-chymotrypsin extracts showed the highest alkyl radical scavenging activities among various carbohydrases and proteases extracts, and the IC_{50} values were 21.13 and 33.23 μg/mL, respectively. We showed that plant extracts have antioxidative activities on alkyl radical. We reported that *Perilla frutescens* var. *crispa* exerts positive effect against alkyl radical and the IC_{50} value of Termamyl was 250.27 μg/mL (36). *Picrorrhiza kurroa* also exhibited capacity against alkyl radical and the IC_{50} value of Maltogenase was 10.66 μg/mL (37). These facts suggest that enzymatic extracts of the leaves might be potential source of alkyl radical scavenger.

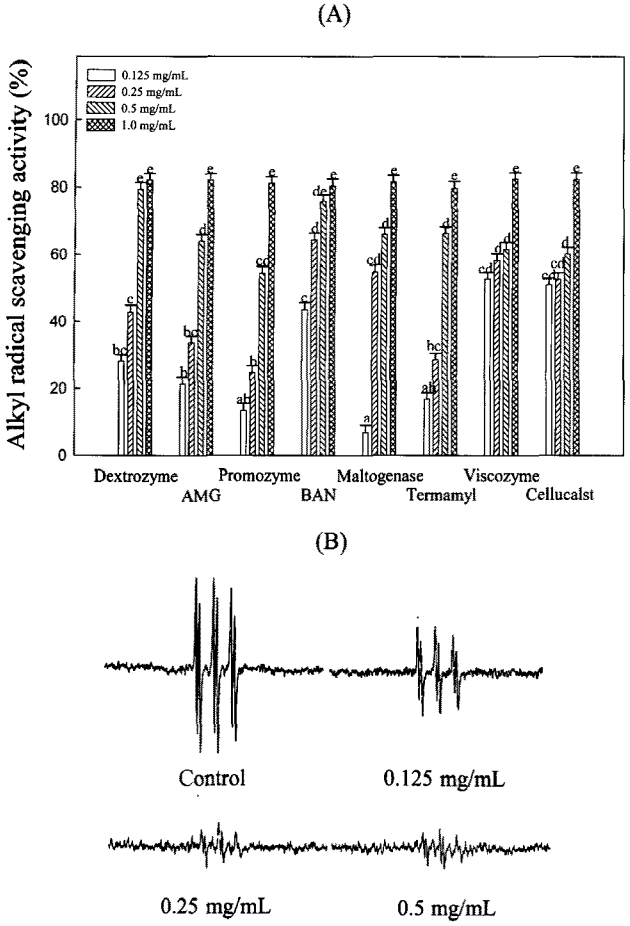

Fig. 5. Alkyl radical scavenging activity of various enzymatic extracts by carbohydratic hydrolysis from leaves of *P. frutescens* (A) and ESR spectra of BAN extracts from leaves of *P. frutescens* (B). Means±SD of determinations were made in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

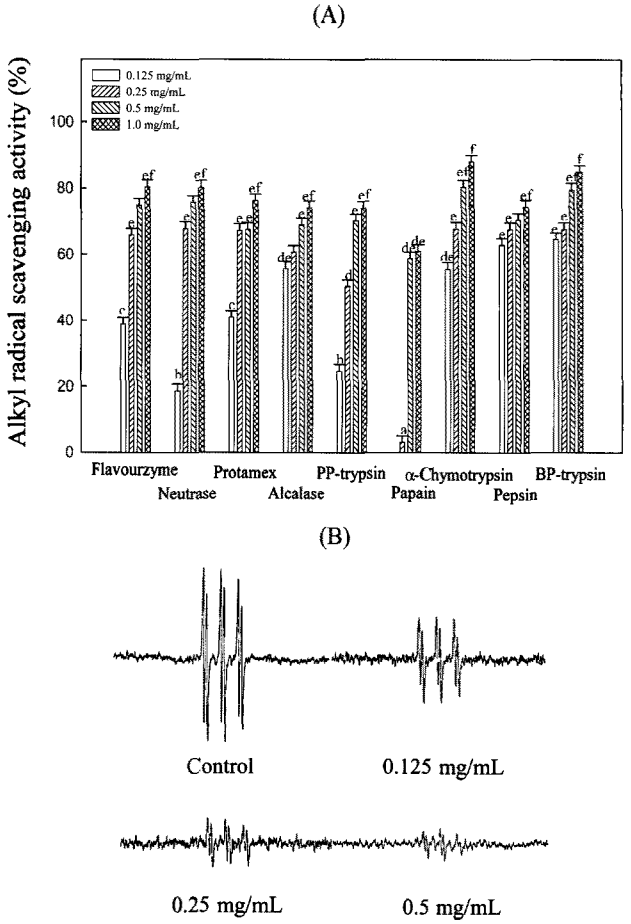

Fig. 6. Alkyl radical scavenging activity of various enzymatic extracts by proteolytic hydrolysis from leaves of *P. frutescens* (A) and ESR spectra of α-chymotrypsin extracts from leaves of *P. frutescens* (B). Means±SD of determinations were made in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

Protective effect of the enzymatic extracts from leaves of *P. frutescens* against hydroxyl radical induced DNA damage The antioxidant effect of enzymatic extracts from the leaves was also evaluated using a protection effect on free radical induced plasmid pBR 322 DNA damage *in vitro*. DNA was broken into 3 forms supercoiled (SC), open circular (OC), and linear form (Linear) when exposed to hydroxyl radical derived from a Fenton reaction. In the present study, Celluclast and pepsin extracts were selected to investigate protective effects on hydroxyl radical-induced DNA damage because the 2 extracts were the highest hydroxyl radical scavenging activities among various carbohydrases and proteases extracts. The protective effects of Celluclast and pepsin extracts from the leaves on free radical-induced DNA damage were shown in Fig. 7. The SC form in DNA was completely reduced under the treatment of hydroxyl radical generated from a Fenton reaction (Lane 2) compared with plasmid DNA control (Lane 1). According to the results, Celluclast and pepsin extracts from the leaves at concentrations in the range of 0.19-0.75 mg/mL protected free radical-induced DNA damage (Lane 3-5).

Neuroprotective effect the enzymatic extracts from leaves of *P. frutescens* against H_2O_2 Due to its strong reactivity with biomolecules, ·OH is probably capable of doing more damage to biological systems than any other ROS (38). Therefore, Celluclast and pepsin extracts which have the highest hydroxyl radical scavenging activity were selected to investigate of the neuroprotective effect against H_2O_2. The neuroprotective effect of Celluclast and pepsin extracts from the leaves was determined with sub-G1 analysis by a flow cytometer. The cells were treated with the extracts from the leaves prior 1 mM H_2O_2 for 24 hr.

There was no effect in the Celluclast extracts from the leaves (data not shown). In the pepsin extracts from the leaves, the percentages of apoptotic cells observed 65.28% at 1 mM H_2O_2, while the percentages of the pepsin extracts treated cells showed 46.53 and 33.57% at 1 and 2 mg/mL, respectively (Fig. 5). These results indicate that the pepsin extracts from the leaves protect neuronal cells against H_2O_2-induced oxidative damage.

Nowadays, there is a continuing search for better and more effective antioxidants. There is a close association between chronic inflammation and cancer. Evidence for

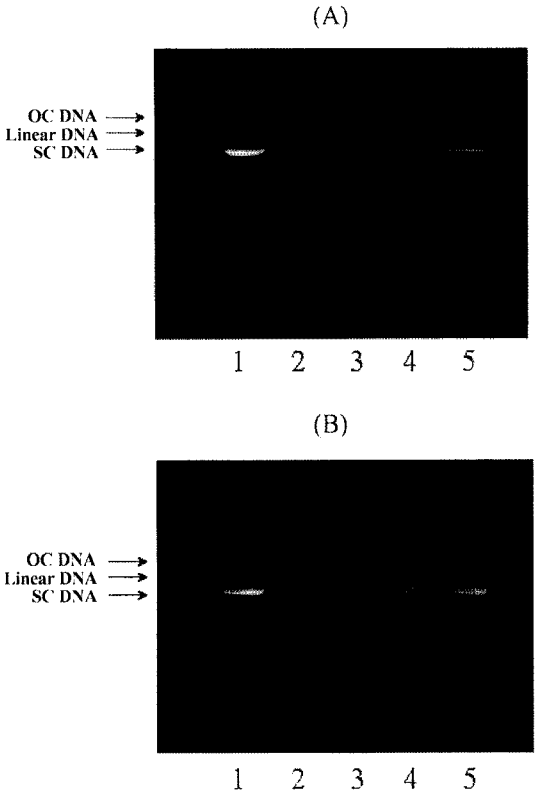

Fig. 7. Agarose gel electrophoretic patterns of plasmid DNA breaks by ·OH generated from a Fenton reaction in the presence of Celluclast (A) and pepsin (B) extracts from leaves of *P. frutescens*. Lane 1, no addition (plasmid DNA control); lane 2, $FeSO_4$ and H_2O_2 (DNA damage control); lane 3-5, $FeSO_4$ and H_2O_2 in the presence of Celluclast or pepsin extracts from leaves of *P. frutescens* with concentrations of 0.19, 0.38, and 0.75 mg/mL, respectively.

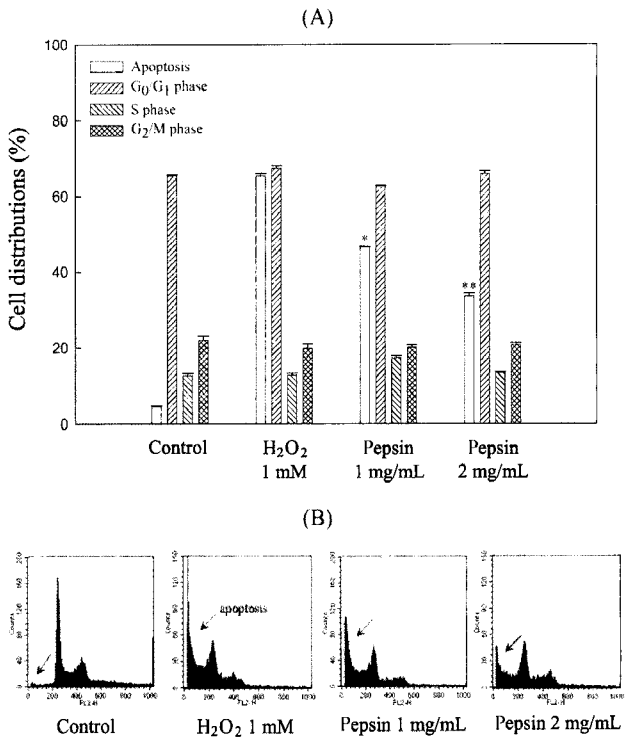

Fig. 8. Cell death and cell cycle of PC-12 after treating the pepsin extracts from leaves of *P. frutescens* prior H_2O_2 treatment. The cells were treated with 1 and 2 mg/mL of the pepsin extracts from leaves of *P. frutescens* prior 1 mM H_2O_2 for 24 hr (A) and the histogram (B). Means±SD of determinations were made in triplicate experiments. $*p<0.05$ and $**p<0.01$ are significantly different as analyzed by paired *t*-test compared DNA damage with sample-treated group. The arrow indicate apoptotic peak.

this comes from epidemiological studies, linking reactive species overload diseases to high cancer risk (39). At the molecular level, free radicals produced during chronic inflammation, can induce deleterious gene mutation and post-translational modifications of key cancer-related proteins (40). The pro-cancerous outcome of chronic inflammation increased DNA damage. A critical mechanism toward cancer associated with reactive species overload diseases is the attack of cancer genes, cancer proteins, RNA, and lipids by reactive nitrogen and oxygen species (40-43). Therefore, to diminish these pro-cancerous mechanisms, a key treatment strategy is to reduce the free radical load.

In the present study, we focused on natural water-soluble antioxidants from *P. frutescens* which prepared by enzymatic hydrolysis using different carbohydrate degrading enzymes and proteases, and their antioxidant effects were evaluated in 3 different free radical scavenging assays including DPPH, hydroxyl, and alkyl radical.

In the earlier reports, the leaves have been known for several biological activities (13-19) and cellular lysosomal enzyme of hot-water extracts from the leaves displayed the more activity than methanol and ethanol extracts (41). However there were no studies about radical scavenging activity by using ESR spectroscopy and protective effects of DNA and neuronal cells of enzymatic extracts from the leaves. Therefore we prepared enzymatic extracts from the leaves by various enzymes to take water soluble extracts, useful oligosaccharides, and peptides. The antioxidative activity of the enzymatic extracts was investigated as a radical scavenger on DHHP, hydroxyl, and alkyl radical. The components in the leaves for the bioactivities are under investigation. Further study is required for identification of antioxidant compounds from enzymatic extracts of the leaves.

In this research, the enzymatic extracts from the leaves showed the neuroprotective effect on H_2O_2-induced cell damage and the protective effect on hydroxyl radical-induced DNA damage. In addition, the enzymatic extracts from the leaves scavenged various free radicals such as DPPH radical, hydroxyl radical, and alkyl radical. Therefore, the enzymatic extracts from the leaves might be used useful natural antioxidant.

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References

1. Sheu SS, Nauduri D, Anders MW. Targeting antioxidants to mitochondria: A new therapeutic direction. Biochim. Biophys. Acta 1762: 256-265 (2006)
2. Cashman JR. Monoamine oxidase and flavin-containing monooxygenases. pp. 69-96. In: Biotransformation. Guengerich FP (ed). Elsevier, New York, NY, USA (1997)
3. Muramatsu H, Kogawa K, Tanaka M, Okumura K, Nishihori Y, Koike K, Kuga T, Niitsu Y. Superoxide dismutase in SAS human tongue carcinoma cell line is a factor defining invasiveness and cell motility. Cancer Res. 55: 6210-6214 (1995)
4. Leanderson P, Faresjo AO, Tagesson C. Green tea polyphenols inhibit oxidant-induced DNA strand breakage in cultured lung cells. Free Radical Bio. Med. 23: 235-242 (1997)
5. Sussman MS, Bulkley GB. Oxygen-derived free radicals in reperfusion injury. Method Enzymol. 186: 711-723 (1990)
6. Debashis DD, Bhattacharjee BM, Banerjee RK. Hydroxyl radical is the major causative factor in stress-induced gastric ulceration. Free Radical Bio. Med. 23: 8-18 (1997)
7. Devinder S, Rajnendrapal K, Vikas C, Kanwaljit C. Antioxidants in the prevention of renal disease. J. Med. Food 9: 443-450 (2006)
8. Vajragupta O, Boonchoong P, Wongkrajang Y. Comparative quantitative structure-activity study of radical scavengers. Bioorg. Med. Chem. 8: 2617-2628 (2000)
9. Facchinetti F, Dawson VL, Dawson TM. Free radicals as mediators of neuronal injury. Cell. Mol. Neurobiol. 18: 667-682 (1997)
10. Prasad KN, Cole WC, Hovland AR, Prasad KC, Nahreini P, Kumar B, Edwards-Prasad J, Andreatta CP. Multiple antioxidants in the prevention and treatment of neurodegenerative disease: Analysis of biologic rationale. Curr. Opin. Neurol. 12: 761-770 (1999)
11. Delanty N, Dichter MA. Antioxidant therapy in neurologic disease. Arch. Neurol.-Chicago 57: 1265-1270 (2000)
12. Izawa K. Colored Picture Book of Medicinal Herbs. Vol 1. Shufunotomo, Tokyo, Japan. pp. 128-130 (1991)
13. Kim MK, Lee HS, Kim Ej, Won NH, Chi YM, Kim BC, Lee KW. Protective effect of aqueous extract of *Perilla frutescens* on *tert*-butyl hydroperoxide-induce dxidative hepatotoxicity in rats. Food Chem. Toxicol. 45: 1738-1744 (2007)
14. Makino T, Furuta Y, Wakushima H, Fujii H, Saito K, Kano Y. Anti-allergic effect of *Perilla frutescens* and its active constituents. Phytother. Res. 17: 240-243 (2003)
15. Makino T, Furuta A, Fujii H, Nakagawa T, Wakushima H, Saito K, Kano Y. Effect of oral treatment of *Perilla frutescens* and its constituents on type-I allergy in mice. Biol. Pharm. Bull. 24: 1206-1209 (2001)
16. Ryu JH, Son HJ, Lee SH, Sohn DH. Two neolignans from *Perilla frutescens* and their inhibition of nitric oxide synthase and tumor necrosis factor-alpha expression in murine macrophage cell line RAW 264.7. Bioorg. Med. Chem. Lett. 25: 649-651 (2002)
17. Banno N, Akihisa T, Tokuda H, Yasukawa K, Higashihara H, Ukiya M, Watanabe K, Kimura Y, Hasegawa J, Nishino H. Triterpene acids from the leaves of *Perilla frutescens* and their anti-inflammatory and antitumor-promoting effects. Biosci. Biotech. Bioch. 68: 85-90 (2004)
18. Ueda H, Yamazaki C, Yamazaki M. Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens*. Biol. Pharm. Bull. 25: 1197-1202 (2002)
19. Chun HS, Hur SY, Kim BH, Kim CJ. Macrophage stimulating activity of water extract from defatted perilla seeds (*Perilla frutescens* var. *japonica*). Food Sci. Biotechnol. 11: 684-689 (2002)
20. Je JY, Park PJ, Kim SK. Free radical scavenging properties of heterochitooligosaccharides using an ESR spectroscopy. Food Chem. Toxicol. 42: 381-387 (2004)
21. Park PJ, Heo SJ, Park EJ, Kim SK, Byun HG, Jeon BT, Jeon YJ. Reactive oxygen scavenging effect of enzymatic extracts from *Sargassum thunbergii*. J. Agr. Food Chem. 53: 6666-6672 (2005)
22. Kim SK, Kim YT, Byun HG, Park PJ, Ito H. Purification and characterization of antioxidative peptides from bovine skin. J. Biochem. Mol. Biol. 34: 214-219 (2001)
23. Rajapakse N, Mendis E, Jung WK, Je JY, Kim SK. Purification of radical scavenging peptide from fermented mussel sauce and its antioxidant properties. Food Res. Int. 38: 175-182 (2005)
24. Park PJ, Je JY, Kim SK. Free radical scavenging activity of chitooligosaccharides by electron spin resonance spectrometry. J. Agr. Food Chem. 51: 4624-4627 (2003)
25. Ou Syi, Jackson GM, Jiao X, Chen J, Wu JZ, Huang XS. Protection against oxidative stress in diabetic rats by wheat bran feruloyl oligosaccharides. J. Agr. Food Chem. 55: 3191-3195 (2007)
26. Megias C, Pedroche J, Yust MM, Giron-Calle J, Alaiz M, Millan F, Vioque J. Affinity purification of copper-chelating peptides from sunflower protein hydrolysates. J. Agr. Food Chem. 55: 6509-6514 (2007)
27. Heo SJ, Jeon YJ, Lee J, Kim HT, Lee KW. Antioxidant effect of enzymatic hydrolyzate from a Kelp, *Ecklonia cava*. Algae 18: 341-347 (2003)
28. Figueroa-Espinoza MC, Poulsen C, Soe JB, Zargahi MR, Rouau X. Enzymatic solubilization of arabinoxylans from native, extruded, and high-shear-treated rye bran by different endo-xylanases and other hydrolyzing enzymes. J. Agr. Food Chem. 52: 4240-4249 (2004)
29. Sorensen HR, Pedersen S, Vikso-Nielsen A, Meyer AS. Efficiencies of designed enzyme combinations in releasing arabinose and xylose from wheat arabinoxylan in an industrial ethanol fermentation residue. Enzyme Microb. Tech. 36: 773-784 (2005)
30. Lowry OH, Rosebrough AL, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagent. J. Bio. Chem. 193: 165-175 (1951)
31. Dubois M, Gilles JK, Hamilton PA, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350-356 (1956)
32. Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. Free Radical Bio. Med. 21: 895-902 (1996)
33. Rosen GM, Rauckman EJ. Spin trapping of superoxide and hydroxyl radicals. pp. 412-430. In: Methods in Enzymology. Packer L (ed). Academic Press, Orlando, FL, USA (1984)
34. Yeung SY, Lan WH, Huang CS, Lin CP, Chan CP, Chang MC. Scavenging property of three cresol isomers against H_2O_2, hypochlorite, superoxide, and hydroxyl radicals. Food Chem. Toxicol. 40: 1403-1413 (2002)
35. Calliste CA, Trouillas P, Allais DP, Duroux JL. Castanea. *Castanea sativa Mill.* leaves as new sources of natural antioxidant: An electronic spin resonance study. J. Agr. Food Chem. 53: 282-288 (2005)
36. Kim EK, Lee SJ, Jeon YJ, Ahn CB, Song MD, Park TK, Moon SH, Jeon BT, Shahidi F, Park PJ. Antioxidant effect and DNA protective effect of various enzymatic extracts from *Perilla frutescens* var. *crispa*. J. Food Lipids 14: 335-349 (2007)
37. Choi SH, Kim EK, Lee SJ, Moon SH, Lee CH, Jeon BT, Park IS, Park TK, Park PJ. Antioxidant activity and protective effect on hydroxyl radical-induced DNA damage of enzymatic extracts from *Prcrorrhiza kurroa*. J. Food Biochem. 32: in press (2008)
38. Halliwell B. Antioxidant defense mechanisms; from the beginning to end (of the beginning). Free Radical Res. 31: 261-272 (1999)
39. Hofseth LJ, Ying L. Identifying and defusing weapons of mass inflammation in carcinogenesis. Bio. Biophy. Acta 1765: 74-84 (2006)
40. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. Nat. Rev. Cancer 3: 276-285 (2003)
41. Kwon KH, Kim KI, Jun WJ, Shin DH, Cho HY, Hong BS. *In vitro* and *in vivo* effects of macrophage-stimulatory polysaccharide from leaves of *Perilla frutescens* var. *crispa*. Biol. Pharm. Bull. 25: 367-371 (2002)
42. Lee SH, Jew SS, Chang PS, Hong IJ, Hwang ES, Kim KS, Kim KT. Free radical scavenging effect and antioxidant activities of barley leaves. Food Sci. Biotechnol. 12: 268-273 (2003)
43. Choi JM, Han J, Yoon BS, Chung JH, Shin DB, Lee SK, Hwang JK, Ryang R. Antioxidant properties of tannic acid and its inhibitory effects on paraquat-induced oxidative stress in mice. Food Sci. Biotechnol. 15: 728-734 (2006)