Protective Effects of Broccoli (*Brassica oleracea*) and Its Active Components against Radical-Induced Oxidative Damage

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(Received May 31, 2004)

Summary The radical scavenging effect and protective potential from oxidative damage by radical generator, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), in renal epithelial LLC-PK₁ cell of broccoli (*Brassica oleracea*) were investigated and identified the active components under the bioassay-linked fractionation method. The MeOH extract, and fractions of CH₂Cl₂, BuOH and H₂O from broccoli showed the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect in a dose-dependent manner. In addition, they exerted the protective effect against LLC-PK₁ cellular damage induced by AAPH dose-dependently. In particular, the BuOH fraction was evaluated as the most active fraction, indicating that the BuOH fraction contains the active components with antioxidative capacity. Employing a bioassay-linked fractionation method, the active principles were isolated and characterized as 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose from the BuOH fraction. These two compounds from broccoli displayed potent antioxidant effects against the DPPH radical, showing the IC₅₀ values of 5.18 and 7.52 µg/mL, respectively. Moreover, the compounds significantly and dose-dependently recovered cell viability lowered by AAPH treatment, suggesting the protective roles from cellular oxidative damage. The present study suggests that broccoli has excellent antioxidative potential and the hydroxycinnamic acid esters from broccoli, 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose, are considered as the active components with antioxidative effect.

Key Words broccoli, antioxidative activity, 1,2-disinapoylgentiobiose, 1-sinapoyl-2-feruloylgentiobiose

Free radicals and other reactive species are considered to be important causative factors in the development of various diseases and the aging process (1–4). This relationship has led to considerable interest in the search for antioxidants to scavenge free radicals and elevate the defense system. Although several synthetic antioxidants have been suggested for the prevention and treatment of diseases, various side effects and toxicities have become an issue. Therefore, the natural antioxidants from foods have attracted much attention and great effort has been made to search for safe and effective therapeutic agents for oxidative stress-related diseases.

Compelling evidence indicates that increased consumption of dietary antioxidants or vegetables with antioxidant properties may contribute to the improvement of the quality of life by delaying onset and reducing the risk of degenerative diseases (5–7). Epidemiological studies have shown that increased consumption of

fruits and vegetables containing high levels of phytochemicals has been recommended to prevent chronic diseases related to oxidative stress in the human body (8–11). In particular, a diet rich in cruciferous vegetables, such as broccoli, cabbage, brussels sprouts, cauliflower and kale, can reduce the risk from chronic degenerative diseases (12, 13). However, the antioxidative activities of these vegetables and the components with activity have not been clearly demonstrated.

Therefore, we investigated the free radical scavenging effect and antioxidative capacity of vegetables under in vitro and cellular models of oxidative stress (data not shown), and broccoli (*Brassica oleracea*) among them has been evaluated as the one with excellent antioxidative potential. Broccoli naturally contains many antioxidants, including carotenoids, tocopherol, ascorbic acid and flavonoids (14, 15); thus the antioxidative activity has been suggested. Therefore, the present study was focused on the radical scavenging effect of broccoli under in vitro and cellular system, and the active components of broccoli have been identified using the bioassay-linked fractionation method and the antioxidative

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activity verified.

MATERIALS AND METHODS

Materials. Broccoli was purchased at a market in Korea. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) was purchased from Amresco (Solon, Ohio, USA). The LLC-PK₁ cell, a porcine renal epithelial cell, was provided by ATCC (Manassas, VA, USA). Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY). NMR spectra were measured on a Bruker Avance 500 spectrometer (Bruker, Ettlingen, Germany, 500 MHz for ¹H). The Fab-MS was recorded on a Jeol JMS-700 mass spectrometer (Jeol, Tokyo, Japan).

Extract preparation and fractions from broccoli. The dried broccoli (570 g) was refluxed with MeOH for 3 h. The organic solvent was removed in a vacuum to give 20 g of MeOH extract. The MeOH extract was dissolved in water and further partitioned in succession with H₂O, CH₂Cl₂ and *n*-BuOH, affording 8.4, 4.5 and 5.5 g of the respective fractions.

Bioactivity-guided fractionation and comparison of radical scavenging activity. From the BuOH fraction, activity-guided isolation by silica gel chromatography eluted with chloromethane-MeOH (gradient elution, 10 : 1 → 1 : 1) gave 7 fractions, and the DPPH radical scavenging activity of the 7 fractions was measured and compared.

Isolation and identification of active components. The most active fraction among the 7 fractions, fraction 5, was further purified by silica gel chromatography using an EtOAc-MeOH (10 : 1) system. The two major components were isolated and their chemical structures were characterized by spectroscopic techniques of mass and NMR. Compound **I** (5 mg) and **II** (6 mg) were identified as 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose, respectively (16), and their chemical structures are illustrated in Fig. 1.

(1) 1,2-Disinapoylgentiobiose: Yellow crystal (MeOH), C₃₄H₄₂O₁₉, FAB-MS *m/z*: 777 [M+Na]⁺, ¹H-NMR (500 MHz, DMSO-d₆): 7.63 (1H, d, *J*=15.8 Hz, H-7'), 7.62 (1H, d, *J*=15.8 Hz, H-7), 6.88 (2H, s, H-2', 6'), 6.85 (2H, s, H-2, 6), 6.40 (1H, d, *J*=15.8 Hz, H-8'), 6.32 (1H, d, *J*=15.8 Hz, H-8), 3.85 (3H, s, OCH₃'), 3.83 (3H, s, OCH₃'), 5.77 (1H, d, *J*=8.83 Hz, glu-1), 5.08 (1H, dd, *J*=8.8, 8.8 Hz, glu-2), 4.35 (1H, d, *J*=7.9 Hz, glu-1'), 4.11 (1H, d, *J*=10.2 Hz, glu-6b), 3.20 (1H, dd, *J*=7.9, 8.7 Hz, glc-3'), 3.12 (1H, dd, *J*=7.9, 8.7 Hz, glc-2'), ¹³C-NMR (125 MHz, DMSO-d₆): 165.0 (C-9'), 164.2 (C-9), 147.5 (C-3, 3', 5, 5'), 146.8 (C-7'), 145.5 (C-7), 138.3 (C-4'), 137.9 (C-4), 123.6 (C-1), 123.5 (C-1'), 114.1 (C-8'), 113.1 (C-8), 106.1 (C-2', 6'), 105.5 (C-2, 6), 102.6 (glc-1'), 91.5 (glc-1), 76.5 (glc-5'), 76.2 (glc-5), 75.9 (glc-3'), 73.2 (glc-3), 72.9 (glc-2), 72.1 (glc-2'), 69.4 (glc-4'), 69.0 (glc-4), 67.2 (glc-6), 60.5 (glc-6'),

1,2-disinapoylgentiobiose

1-sinapoyl-2-feruloylgentiobiose

Fig. 1. Chemical structures of active components isolated from broccoli.

55.6 (OCH₃'), 55.5 (OCH₃).

(2) 1-Sinapoyl-2-feruloylgentiobiose: Yellow crystal (MeOH), C₃₃H₄₀O₁₈, FAB-MS *m/z*: 747 [M+Na]⁺, ¹H-NMR (500 MHz, DMSO-d₆): 7.62 (1H, d, *J*=15.8 Hz, H-7'''), 7.60 (1H, d, *J*=15.8 Hz, H-7), 7.30 (1H, d, *J*=2.0 Hz, H-2'''), 7.09 (1H, dd, *J*=2.0, 7.9 Hz, H-6'''), 7.02 (2H, s, H-2, 6), 6.79 (1H, d, *J*=7.9 Hz, H-5'''), 6.48 (1H, d, *J*=15.8 Hz, H-8), 6.48 (1H, d, *J*=15.8 Hz, H-8'''), 3.84 (3H, s, OCH₃'), 3.83 (3H, s, OCH₃'), 5.76 (1H, d, *J*=8.8 Hz, glu-1), 4.92 (1H, dd, *J*=8.8, 8.8 Hz, glu-2), 4.35 (1H, d, *J*=7.9 Hz, glu-1'), 4.07 (1H, d, *J*=11.2 Hz, glu-6b), 3.15 (1H, dd, *J*=7.9, 8.7 Hz, glc-3'), 3.02 (1H, dd, *J*=7.9, 8.7 Hz, glc-2'), ¹³C-NMR (125 MHz, DMSO-d₆) *d*: 165.2 (C-9), 164.4 (C-9'), 148.8 (C-4'), 147.5 (C-3, 5), 147.4 (C-3'), 146.8 (C-7), 145.0 (C-7'), 138.3 (C-4), 124.9 (C-1'), 123.5 (C-1), 122.8 (C-6'), 114.9 (C-5'), 113.6 (C-8'), 113.1 (C-8), 110.6 (C-2'), 106.1 (C-6), 102.5 (glc-1'), 91.5 (glc-1), 76.5 (glc-5'), 76.2 (glc-5), 75.9 (glc-3'), 73.2 (glc-3), 72.8 (glc-2), 72.1 (glc-2'), 69.4 (glc-4'), 69.0 (glc-4), 67.2 (glc-6), 60.5 (glc-6'), 55.6 (OCH₃), 55.0 (OCH₃).

DPPH radical scavenging activity. One hundred microliters of an EtOH solution of the sample (the control used 100 μL ethanol instead of the sample) was added to microwells followed by 100 μL of 120 μM DPPH in EtOH, according to the method of Hatano et al. (17). After gentle mixing and 30 min of standing at room temperature, the DPPH radical level was measured with a microplate reader, model SPECTRAMax 340PC (Molecular Devices, Sunnyvale, CA, USA). The

antioxidant activity was expressed as the inhibition rate of the DPPH radical or the IC₅₀ (concentration in $\mu\text{g/mL}$ required to inhibit DPPH radical formation by 50%) determined from the log-dose inhibition curve.

Experiment with cultured cells. Commercially available LLC-PK₁ cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in culture plates with 5% FBS-supplemented DMEM/F-12 medium. To investigate the protective activity from oxidative damage, we employed the AAPH-induced cellular oxidative model (18). After confluence had been reached, the cells were seeded into 96-well culture plates at 10⁴ cells per well. Two hours later, 1 mM of AAPH was added to all of the wells, and then samples of extract, fractions or the compounds from broccoli were treated in the test wells. The plates were then incubated under routine conditions for 24 h. The proper concentration of AAPH and the incubation time were determined by the preliminary experiment. Fifty microliters of MTT (1 mg/mL) solution was added to the each well. After incubation for 4 h at 37°C, the MTT solution was removed from the medium. The resultant formazan crystals in the renal cells were solubilized with 100 μL of dimethylsulfoxide. The absorbance of each well was then read at 540 nm using a microplate reader (model SPECTRAMax 340PC, Molecular Devices).

Statistical analysis. The results for each group were expressed as mean $\pm$ SD values. Data were analyzed by one way ANOVA between control and sample treated groups by SAS (SAS Institute Inc., Cary, NC, USA). Significant differences were determined among groups at $p < 0.05$.

RESULTS

DPPH radical scavenging activity

As shown in Fig. 2, the extract of MeOH, and fractions of CH₂Cl₂, BuOH and H₂O from broccoli showed the DPPH radical scavenging effect in a dose-dependent manner. In particular, the BuOH fraction exerted the

Fig. 2. Effect of MeOH extract (●) and fractions of CH₂Cl₂ (○), BuOH (■) and H₂O (□) from broccoli on DPPH radical.

Table 1. Effect of 7 fractions from BuOH fraction of broccoli on DPPH radical.

Fraction No.	Concentration ($\mu\text{g/mL}$)	DPPH radical scavenging activity (%)
1	10	0
	25	0
	50	5.96 $\pm$ 0.22
	100	12.39 $\pm$ 0.65
2	10	1.83 $\pm$ 0.43
	25	13.76 $\pm$ 0.43
	50	20.18 $\pm$ 1.30
	100	26.15 $\pm$ 0.65
3	10	0.92 $\pm$ 0.43
	25	8.26 $\pm$ 1.30
	50	16.97 $\pm$ 0.65
	100	27.06 $\pm$ 0.65
4	10	1.38 $\pm$ 0.65
	25	13.30 $\pm$ 0.22
	50	25.23 $\pm$ 0.22
	100	44.04 $\pm$ 0.01
5	10	2.75 $\pm$ 0.01
	25	21.10 $\pm$ 0.43
	50	46.33 $\pm$ 1.51
	100	80.73 $\pm$ 0.86
6	10	2.29 $\pm$ 1.08
	25	11.47 $\pm$ 0.22
	50	21.10 $\pm$ 0.01
	100	36.70 $\pm$ 0.01
7	10	0
	25	2.29 $\pm$ 0.65
	50	4.13 $\pm$ 0.22
	100	31.19 $\pm$ 0.43

strongest inhibition effect on DDPH radical generation, showing the inhibition of more than 80% at 50 $\mu\text{g/mL}$ and almost 90% at 100 $\mu\text{g/mL}$. In addition, Table 1 shows the result of DPPH radical scavenging activity linked with fractionation. Among the 7 fractions from the BuOH fraction of broccoli, fraction 5 exerted the strongest activity, showing 46 and 81% of DPPH radical scavenging activities at concentrations of 50 and 100 $\mu\text{g/mL}$, respectively. On the other hand, other fractions showed relatively low DPPH radical scavenging activities.

Protective effect from LLC-PK₁ cellular damage induced by AAPH

Figure 3 shows the protective activity of extract and fractions from broccoli against AAPH-induced cellular damage. The treatment of AAPH resulted in the decrease of LLC-PK₁ cell viability to 61.3% compared with 100% of AAPH-non treated normal cells. On the other hand, the MeOH extract and fractions of CH₂Cl₂, BuOH and H₂O elevated the cell viability significantly. In particular, the BuOH fraction recovered cell viability

Fig. 3. Effect of MeOH extract (●) and fractions of CH₂Cl₂ (○), BuOH (■) and H₂O (□) from broccoli on viability of LLC-PK₁ cells treated with AAPH.

to 80% even at the low concentration of 5 µg/mL, and to more than 90% at 100 µg/mL, so the BuOH fraction revealed the strongest protective activity.

Antioxidative effects of 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose

The DPPH radical-scavenging effect of the active component of broccoli is shown in Table 2. The IC₅₀ values were 5.18 and 7.52 µg/mL of 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose, respectively. In addition, as revealed in Table 3, 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose significantly and dose-dependently recovered the cell

viability lowered by the treatment of AAPH. They increased the cell viability of AAPH treated control cells from 61.3% almost to 90% at 2.5 µg/mL. Moreover, the treatment of 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose at the concentration of 10 µg/mL elevated the cell viability of LLC-PK₁ to nearly normal values.

DISCUSSION

As the negative relationship between antioxidative status and various pathological conditions has been clearly recognized (5, 6), interest has been focused on the health benefits derived from food with antioxidant activity. Biologically active compounds can be found in vegetables and may play a role in reducing the risk of degenerative diseases involved in oxidative stress (19–21). In particular, broccoli is becoming increasingly popular as a potential source of biologically active dietary components including flavonol glycosides, hydroxycinnamic acids and sulfur-containing compounds such as glucosinolates. We investigated the radical scavenging effect and antioxidative potential of extract and fractions of broccoli, and identified its active components. The *in vitro* antioxidative effect has been evaluated as the scavenging activity of the DPPH radical, and antioxidative potential under the cellular system of oxidative stress from AAPH using LLC-PK₁ renal tubular epithelial cells that are susceptible to oxidative stress has also been observed.

To study the reactions of free radicals biologically, a well-designed *in vitro* model system is required. Numerous factors induce oxidative stress, such as irradiation, redox decomposition by metal ions of hydroperoxides or hydrogen peroxide, and thermal decomposition of free radical initiators including peroxides, hyponitrites, and azo compounds. To generate free radicals at a known, constant and well-defined rate, thermal decomposition of free radical initiators is preferred. It has been suggested that this can be achieved by the use of AAPH, one of the hydrophilic azo compounds. AAPH generates free radicals at a constant and measurable rate by its thermal decomposition without biotransformation. The

Table 2. DPPH radical scavenging activity of active components from broccoli.

Compound	IC ₅₀ (µg/mL)
1,2-Disinapoylgentiobiose	5.18±1.11
1-Sinapoyl-2-feruloylgentiobiose	7.52±1.64

Table 3. Effect of active components from broccoli on viability of LLC-PK₁ cells treated with AAPH.

Treatment (µg/mL)	Cell viability (%)	
	1,2-Disinapoylgentiobiose	1-Sinapoyl-2-feruloylgentiobiose
AAPH-treated control	61.3±3.6 ^a	61.3±3.6 ^a
0.5	79.0±4.5 ^b	72.5±1.3 ^b
1	86.9±3.1 ^c	79.4±2.8 ^c
2.5	89.3±2.5 ^c	88.0±1.7 ^d
5	96.9±0.9 ^d	89.1±1.7 ^d
10	100.2±2.5 ^d	96.6±3.4 ^e
Normal	100.0±3.1	

Means with the different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test.

free radicals generated from AAPH react with oxygen molecules rapidly to yield peroxy radicals. The lipid peroxy radicals attack other lipid radicals. This reaction takes place repeatedly with resultant attacks upon various biological molecules, and induces physiochemical alterations and cellular damage (22). Finally, AAPH causes a diverse array of pathological changes. Therefore, an AAPH-intoxication experiment may be a promising assay system for the biological activities of antioxidants. In an appropriate model system, hydrophilic antioxidants can be used to scavenge radicals and suppress the oxidation initiated by AAPH within cells (23, 24). In addition, AAPH administration results in the in vivo damage of biological tissues such as lymphocytes, kidney and liver, leading to pathological conditions including atherosclerosis, ischemia-reoxygenation injury, and inflammatory disease.

The present study also supports that AAPH leads to the decreased viability of LLC-PK₁ renal epithelial cells. Consistent with this result, several reports have documented that AAPH decreased the viability of hepatic cells, neuron and aortic endothelial cells and the treatment with AAPH induced apoptosis in the cells, causing loss of viability (25–27). On the other hand, this study demonstrated that the extract and fractions of broccoli exerted the DPPH radical scavenging effect; in addition, the oxidative damage to LLC-PK₁ cells by AAPH has been recovered with the treatment of broccoli, resulting in the increase of cell viability in a significant and dose dependent manner. In particular, the in vitro and cellular oxidative damage system showed that the BuOH fraction has the strongest activity against free radical generation and oxidative damage. The application of broccoli extract or fractions to LLC-PK₁ cells damaged by AAPH recovered cellular loss caused by peroxy radicals from AAPH, suggesting that the compounds in broccoli, especially in those from the BuOH fraction, may play a vital role in the protection from oxidative damage from AAPH. Furthermore, the bioassay-linked fractionation method identified the compounds with activity as 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose.

Cruciferous vegetables including broccoli contain several classes of compounds that can potentially contribute to antioxidant activity including vitamins, phenols and glucosinolates. Plumb et al. (28) reported that the glucosinolates in cruciferous vegetables including broccoli are unlikely to account for the direct antioxidant effects of cruciferous vegetables. They also suggest that the antioxidant activity of the extracts probably involves the hydroxylated phenol and polyphenol content of cruciferous vegetables, so the excellent antioxidative potential may be attributed to the large amounts of quercetin and kaempferol in broccoli. In the addition to these reports, the present study clearly demonstrates that 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose are also mainly responsible for the radical scavenging effect and protective activity from oxidative damage. These two compounds from broccoli have been reported (15), but their antioxidative capacities

under a cellular damage model have been first suggested in the present study.

The implication of hydroxycinnamic acids in the diet is receiving much interest and they have been considered as the biologically active compounds; however, the evidence has not been clearly supported. Of the biological activities, the antitumor or anticancer activity of hydroxycinnamic acid esters such as ferulic acid has been suggested (29–31). On the other hand, the radical scavenging activity and protective effect from peroxy radicals of 1,2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose have not been reported yet, and other promising potentials have not been studied.

We could also hypothesize that some portion of the compounds are not absorbed in the small intestine, and these pass into the colon where they are broken down by gut microbes on the basis of the reported evidence on the metabolism of flavonoid glycosides in vivo (32, 33). Further study is needed to verify the uptake and absorption, and the metabolism in animals or humans.

Acknowledgments

This work was supported by a Korean Research Foundation Grant (KRF-2004-003-F00044).

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