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Influence of Polyphenol and Ascorbate Oxidases during Cooking Process on the Radical-Scavenging Activity of Vegetables

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The influence of polyphenol oxidase and ascorbate oxidase on radical-scavenging activity and contents of total phenol, chlorogenic acid, and ascorbic acid in vegetables during the cooking process were investigated. In the case of burdock and lettuce, which have a high activity of polyphenol oxidase, the radical-scavenging activity and the content of total phenol and chlorogenic acid decreased drastically within 1 min. In the case of broccoli, however, only a small decrease of radical-scavenging activity was observed, and total phenol and chlorogenic acid decreased almost not at all. The decrease of the activity in broccoli depended on the oxidation of ascorbic acid by ascorbate oxidase. None of these compounds decreased after the enzymes had been inactivated by heating.

Keywords: radical-scavenging activity, polyphenol oxidase, polyphenol, chlorogenic acid, ascorbic acid, ascorbate oxidase

Dietary vegetables contain a wide variety of free radical-scavenging antioxidants such as polyphenols and vitamins (Huang *et al*., 1994; Shahidi & Naczk, 1995). Epidemiological studies have shown that vegetable consumption is associated with a reduced risk of life-style related diseases such as cancer and coronary heart disease (Steinmetz & Potter, 1991; Ames *et al*., 1993; Joshipura *et al*., 1999). Therefore, antioxidants in vegetables have received considerable attention in recent years for their role in human health (Ames *et al*., 1993; Aruoma, 1994). However, the antioxidant activity present in fresh vegetables may be affected by processing and cooking procedures prior to consumption. We recently reported interesting results that cooked vegetables exhibit a higher radical-scavenging activity than fresh ones (Yamaguchi *et al*., 2001). Maeda *et al*. (1992) also reported the same results, and stated that an increase in the radical-scavenging activity of vegetables might be due to the thermal destruction of vegetable cell walls and subcellular compartments which liberates more components, and/or to a thermal chemical reaction which produces more potent radical-scavenging antioxidants. However, there are no data for the apparent reason why cooked vegetables exhibit a higher radical-scavenging activity than fresh ones. We assume that these observations may be due to heat inactivation of oxidative enzymes such as polyphenol oxidase (EC 1.14.18.1) and ascorbate oxidase (EC 1.10.3.3).

When fruits or vegetables are damaged by bruising or cutting, polyphenols are involved in the process known as enzymatic browning (Mayer & Harel, 1979; Murata & Homma, 1998). Polyphenols are oxidized to their corresponding quinones by polyphenol oxidase. These quinones are further polymerized with quinones or amines to form brown pigments. The economic and nutritional loss induced by enzymatic browning is of concern to food processors and researchers. Therefore, numerous studies have been devoted to the biochemical and catalytic properties of polyphenol oxidase and to the inhibition of polyphenol oxidase activity in several fruits and vegetables including apple (Murata *et al*., 1992), peach (Chang *et al*., 2000), banana (Cano *et al*., 1990; Galeazzi *et al*., 1981), potato (Chen *et al*., 1992), mushroom (Rodríguez-López *et al*., 1999; Devece *et al*., 1999), eggplant (Pérez-Gilabert & Carmona, 2000), Chinese cabbage (Nagai & Suzuki, 2001), and lettuce (Fujita *et al*., 1991; Cantos *et al*., 2001). In eggplant, burdock and lettuce, enzymatic browning results from the enzymatic oxidation of chlorogenic acid (5-caffeoylquinic acid), which exists in large quantities (Fujita & Tono, 1988; Fujita *et al*., 1991). Chlorogenic acid, a potent phenolic antioxidant, is the major substrate of polyphenol oxidase (Murata *et al*., 1992). Other antioxidative phenolic compounds are also substrates of polyphenol oxidase (Murata *et al*., 1995; Nagai & Suzuki, 2001; Jiménez & Garcína-Carmona, 1999); however, there are few reports related to the influence of polyphenol oxidase on either polyphenol content or radical-scavenging activity.

Ascorbic acid, the reduced form of vitamin C, is a common component of vegetables and has strong radical-scavenging activity. It is oxidized to dehydroascorbic acid by ascorbate oxidase, after the vegetable is cut and exposed to oxygen. However, dehydroascorbic acid, the oxidized form of vitamin C, has no radical-scavenging activity (Takamura *et al*., 2002).

The purposes of this study were to determine the influence of

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polyphenol oxidase on total phenol and chlorogenic acid contents, as well as the radical-scavenging activity of vegetables during processing and heating. The involvement of ascorbic acid and ascorbate oxidase in the radical-scavenging activity of vegetables was also investigated.

Materials and Methods

Reagents 1,1-Diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, tris (hydroxymethyl) aminomethane (Tris), 2,4-dinitrophenylhydrazine, and acetonitrile (HPLC grade) were obtained from Nacalai Tesque Inc. (Kyoto). Chlorogenic acid, ethanol and methanol (HPLC grade) were obtained from Wako Pure Chemical Industries (Osaka), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Aldrich Chemical Co. (Milwaukee, WI). The water used in this experiment was purified with Milli-Q Labo equipment (Millipore Japan, Tokyo).

Materials Broccoli (*Brassica oleracea* L. var. *italica* PLENCK), burdock (*Arctium lappa* L.) and lettuce (*Lactuca sativa* L.) were purchased from local markets in Nara, Japan. To retain the active compounds and enzyme activities, these vegetables were frozen and broken in liquid nitrogen. Then they were freeze-dried to powder form and stored at −80°C.

Preparation of vegetable extracts The freeze-dried vegetables powder (0.1 g) was added to 2 ml of water, capped and stirred by shaker for 1, 5, 10, and 15 min to simulate cooking process, and freeze-dried immediately. An aliquot (0.1 g) of freeze-dried sample was extracted for 1 min with 3 ml of 90% methanol, and centrifuged at 1500×*g* for 10 min at 4°C. The supernatant was filtered through a 0.45-μm filter (Cosmonice Filter W, 13 mm, Nacalai Tesque Inc.). The resulting filtrate was used for the measurement of radical-scavenging activity, and total phenol, chlorogenic acid, and ascorbic acid contents in non-heated vegetables. To determine the influence of heating, the freeze-dried vegetable powder (0.1 g) was added to 2 ml of boiling water and heated in a water bath for 10 min, then followed by the same procedure as non-heated vegetables.

Measurement of DPPH radical-scavenging activity Radical-scavenging activity was measured according to the DPPH-HPLC method of Yamaguchi *et al.* (1998). An aliquot of sample solution (200 μl) was mixed with 100 mM of Tris-HCl buffer (pH 7.4, 800 μl) and added to 1 ml of 500 μM DPPH in ethanol. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The reaction mixture was then subjected to HPLC analysis.

The HPLC analysis was carried out on a TSKgel Octyl-80Ts column (4.6×150 mm, Tosoh, Tokyo) equipped with a Shimadzu LC-6A pump (Kyoto), a Rheodyne injector fitted with a 20-μl loop and a Shimadzu SPD-10AV UV-VIS detector set at 517 nm at ambient temperature. The mobile phase consisted of methanol/water (70 : 30, v/v), and the flow rate was 1 ml/min. Trolox was used as the control standard and 200 μl of Trolox solution (final concentration of 50 μM) in ethanol was assayed similarly during each run.

The DPPH radical-scavenging activity was evaluated by the difference in peak area of the DPPH radical detected at 517 nm between a blank and a sample. The activity was expressed as μmol of Trolox equivalent per 100 g of fresh vegetable.

Determination of total phenol content Total phenol content was measured according to the methods of Singleton and Rossi (1965) and calculated using gallic acid as standard. The filtrate (200 μl) was added to 800 μl of 7.5 % Na_2CO_3 and 1 ml of Folin-Ciocalteu reagent, and then left for 30 min. Absorbance was measured at 765 nm using a UV-2100PC UV-VIS spectrophotometer (Shimadzu). Total phenol content was expressed as μmol of gallic acid equivalent per 100 g of fresh vegetables.

Determination of chlorogenic acid content Chlorogenic acid was determined by HPLC. The HPLC system was equipped with a Shimadzu LC-10AD pump, a Lichrospher 100 RP-18 column (4.6×250 mm, E. Merck, Darmstadt, Germany), and a Shimadzu SPD-10AV UV-VIS detector. The column was temperature controlled at 40°C. The mobile phase consisted of 100% methanol (A) and 5% formic acid/water (B) and the flow rate was 1 ml/min. The gradient was as follows: 0 min, 5% B; 10 min, 20% B; 25 min, 25% B; 45 min, 30% B; 55 min, 45% B; 80 min, 55% B; 82 min, 95% B. The wavelength of detection was set at 320 nm. The data were expressed as μmol of chlorogenic acid per 100 g of fresh vegetable.

Measurement of ascorbic acid content Ascorbic acid content was determined by HPLC according to the methods of Kishida *et al.* (1992). Briefly, the filtrate (100 μl) was mixed with or without 0.2% 2,6-dichloroindophenol (50 μl), 1% stannous chloride in 5% metaphosphoric acid solution (50 μl) and 2% 2,4-dinitrophenylhydrazine (120 μl) in 4.5 M sulfuric acid. The mixture was incubated in a water bath for 3 h at 37°C, and then ethyl acetate (1 ml) and water (1 ml) were added. After vortexing, the samples were centrifuged (1500×*g*, 4°C) for 5 min, and 300 μl of the ethyl acetate layer was removed and dried under nitrogen gas. The resulting residue was dissolved in 200 μl of acetonitrile and used for HPLC analysis.

HPLC analysis was carried out on a Cosmosil 5C_{18}-AR-II column (4.6×250 mm, Nacalai Tesque Inc.) with a Shimadzu SPD-10AV UV-VIS detector set at 505 nm and a Rheodyne injector fitted with a 20-μl loop. The mobile phase consisted of acetonitrile/water (50 : 50, v/v) adjusted to pH 3.5 with 0.1% triethylamine and phosphoric acid. The flow rate was 1 ml/min.

Ascorbic acid content was calculated from the difference between the values of the sample with and without 2,6-dichloroindophenol. The data were expressed as mg of ascorbic acid per 100 g of fresh vegetable.

Enzyme extraction Freeze-dried vegetable powder (0.1 g) was extracted with 0.1 mM phosphate buffer (3 ml) for 30 min in an ice bath. The extract was centrifuged at 1500×*g*, 4°C for 10 min. The supernatant was filtered through a 0.45-μm filter (Cosmonice Filter W, 13 mm, Nacalai Tesque Inc.) and was used for measurement of polyphenol oxidase and ascorbate oxidase activities.

Measurement of enzyme activity Polyphenol oxidase activity was measured, using chlorogenic acid as the substrate, according to the spectrophotometric method of Fujita and Tono (1988). The reaction solution consisted of 1.6 ml of 0.1 mM phosphate buffer (pH 6.0), 200 μl of 0.5 mM chlorogenic acid, and 200 μl of the enzyme solution. One unit of polyphenol oxidase was defined as a decrease in absorbance, at 325 nm at 30°C, of 0.01 per min per gram of fresh weight of vegetable.

Ascorbate oxidase activity was measured, using ascorbic acid as the substrate, according to the spectrophotometric method of Yamamoto and Ôba (1999). The reaction solution consisted of

Table 1. Radical-scavenging activity and contents of total phenol, chlorogenic acid, and ascorbic acid in vegetables.

Vegetable	Radical-scavenging activity	Total phenol	Chlorogenic acid	Ascorbic acid
	(μmol Trolox eq./100 g)	(μmol gallic acid eq./100 g)	(μmol/100 g)	(mg/100 g)
Burdock	1851.6±137.7[a)]	830.6±47.7	358.1±29.7	nd[b)]
Lettuce	209.0±14.9	291.9±15.2	10.7±0.4	1.6±0.4
Broccoli	317.2±9.3	600.7±79.6	3.3±0.2	19.7±1.8

[a)] The values are the means±SD for three determinations.
[b)] nd, not detected.

Table 2. Polyphenol oxidase and ascorbate oxidase activity of vegetables.

Vegetable	Enzyme activity	
	Polyphenol oxidase	Ascorbate oxidase
	(unit/min/g fresh weight)	
Burdock	32.4±3.3[a)]	14.5±2.0
Lettuce	149.2±5.0	9.9±2.0
Broccoli	tr[b)]	29.4±3.1

[a)] The values are the means±SD for three determinations.
[b)] tr, trace amounts.

1.6 ml of 0.1 mM phosphate buffer (pH 6.0), 200 μl of 0.5 mM ascorbic acid, and 200 μl of the enzyme solution. One unit of ascorbate oxidase was defined as a decrease in absorbance, at 265 nm at 30°C, of 0.01 per min per gram of fresh weight of vegetable.

Results and Discussion

It is interesting that the radical-scavenging activity of many vegetables increased after heating (Yamaguchi *et al*., 2001; Maeda *et al*., 1992). In general, the purpose of heating of vegetables is to obtain softness, flavor, *etc*. During the heating, polyphenol oxidase and ascorbate oxidase are inactivated by the high temperature. This study was carried out to test the hypothesis about the inactivation of enzymes related with higher radical-scavenging activity of cooked vegetables.

First of all, the radical-scavenging activity, total phenol, chlorogenic acid, and ascorbic acid contents of burdock, lettuce, and broccoli were measured (Table 1). These three vegetables are respective representatives of the root, leafy, and stalk vegetables consumed frequently in Japan.

Among them, burdock had the highest radical-scavenging activity (1851.6 μmol Trolox eq./100 g) and chlorogenic acid content (358.1 μmol/100 g); its radical-scavenging activity was 9- and 6-fold higher than lettuce and broccoli, respectively. The highest total phenol content was also found in burdock (830.6 μmol gallic acid eq./100 g). Wang *et al*. (2001) reported that burdock contains chlorogenic acid, 1,5-dicaffeoylquinic acid, and 1,5-dicaffeoyl-3-succinylquinic acid, with the predominant polyphenol among 13 kinds of burdock being chlorogenic acid. In this study, the predominant polyphenol found in burdock was also chlorogenic acid (data not shown), and the concentration was 33- and 109-fold higher than lettuce and broccoli, respectively. The calculated contribution of chlorogenic acid to the radical-scavenging activity is 53, 14 and 3% in burdock, lettuce and broccoli, respectively. Therefore, the high activity in burdock is derived from its high chlorogenic acid content. Hisaminato *et al*. (2001) reported that lettuce contains chlorogenic acid, caffeoyltartaric acid, dicaffeoyltartaric acid, and 3,5-dicaffeoylquinic acid, with the predominant polyphenol being dicaffeoyltartaric acid. The radical-scavenging activity of lettuce is derived from not only chlorogenic acid but also other phenolic compounds. In the current study, the amount of ascorbic acid was 1.6 mg/100 g in lettuce and 19.7 mg/100 g in broccoli. Thus, the calculated contribution of ascorbic acid to radical-scavenging activity is 4 and 32% in these two vegetables. Ascorbic acid was not detected in burdock. We previously reported that the calculated contribution of ascorbic acid to the radical-scavenging activity is 22, 87, and 2% in fresh material of lettuce, broccoli, and burdock, respectively (Yamaguchi *et al*., 2001). There were some differences in ascorbic acid contribution between freeze-dried and fresh vegetables.

Polyphenol oxidase and ascorbate oxidase activities in the vegetables are shown in Table 2. The highest polyphenol oxidase activity was found in lettuce, while little of this activity was found in broccoli. On the other hand, broccoli showed the highest ascorbate oxidase activity, which was 2 and 3-fold higher than burdock and lettuce. Burdock has ascorbate oxidase activity (14.5 unit/min/g fresh weight) in spite of having no ascorbic acid. This result is not strange because Ôba (1996) reported that there was no correlation between ascorbate oxidase activity and the ascorbic acid content of fresh vegetables.

Polyphenol oxidase and ascorbate oxidase are activated after vegetables are cut and exposed to oxygen. These oxidases oxidized polyphenol and ascorbic acid, and lost their radical-scavenging activity (Takamura *et al*., 2002). The heating of vegetables, in contrast, can inactivate these enzymes and thus inhibits the loss of polyphenols and ascorbic acid. Therefore, we investigated the change in radical-scavenging activity and total phenol, chlorogenic acid, and ascorbic acid content of vegetables during

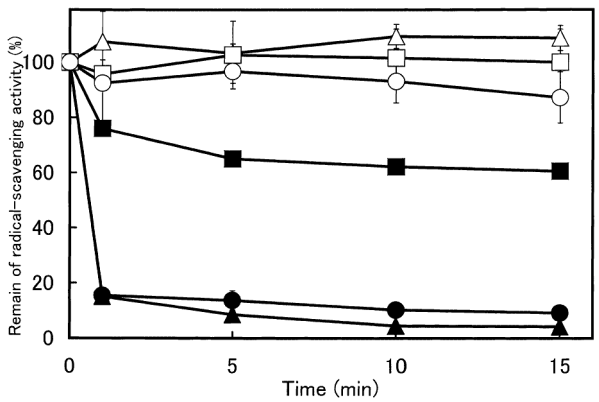

Fig. 1. Effects of heating on the radical-scavenging activity during cooking process of vegetables. ▲, burdock; ●, lettuce; ■, broccoli; △, heated burdock; ○, heated lettuce; □, heated broccoli.

processing using a freeze-dried vegetable powder. Figure 1 shows the change in the radical-scavenging activity of the three vegetables. The radical-scavenging activity of all heated vegetables remained constant during the 15 min-processing period, while the radical-scavenging activity of non-heated vegetables decreased during processing. The activity of unheated burdock, lettuce and broccoli was 278.8, 32.3, and 240.1 μmol Trolox eq./100 g after 1 min, respectively. Thus, the activity of burdock and lettuce decreased drastically to 15% after 1 min, while that of broccoli decreased gradually and remained at about 60% after 15 min.

Figure 2 shows the change in the total phenol content of the three vegetables. The phenol content of all heated vegetables and non-heated broccoli remained constant after 15 min of processing. However, the total phenol content remaining in unheated burdock and lettuce was 266.9 (32%) and 141.2 (48%) μmol gallic acid eq./100 g after 1 min, respectively. Further processing did not affect the total phenol content of lettuce, while a small decrease in this content was observed in broccoli. Some phenolic compounds are known to be substrates of oxidation by polyphenol oxidase (Murata *et al*., 1995; Nagai & Suzuki, 2001). It is also reported that quercetin was oxidized directly by polyphenol oxidase (Jiménez & Garcína-Carmona, 1999). The decrease of total phenol content is probably due to the oxidation by polyphenol oxidase in vegetables.

The change in the chlorogenic acid content is shown in Fig. 3; in all heated vegetables and non-heated broccoli, it was almost constant during 15 min of processing. Interestingly, these results are similar to those for total phenol. Marked decreases in chlorogenic acid in non-heated burdock and lettuce were observed. The content of chlorogenic acid was 49.9 μmol/100 g in burdock after 1 min. Strikingly, no chlorogenic acid was detected in lettuce after 1 min. The remaining percentage of chlorogenic acid was lower than the remaining total phenol content in both lettuce and burdock. Chlorogenic acid is the substrate for enzymatic browning by polyphenol oxidase (Murata *et al*., 1992) and is decreased by polyphenol oxidase (Shahidi & Naczk, 1995). Burdock and lettuce have strong polyphenol oxidase activity (Table 2), which is the reason the content of chlorogenic acid in burdock and lettuce decreased remarkably.

Figure 4 shows the change in ascorbic acid content of the vegetables except for burdock. In the case of lettuce, ascorbic acid was lost completely after 1 min. The remaining percentage of ascorbic acid in broccoli was 34, 11 and 5% after 1, 5, and 10 min, respectively, and none was left after 15 min. Degradation of ascorbic acid in broccoli is considered to be due to the strong activity of ascorbate oxidase (Table 2). Yamamoto and Ôba (1999) reported that dehydroascorbic acid content in cut vegetables was higher than in fresh vegetables because of the oxidation of ascorbic acid by ascorbate oxidase.

Ascorbic acid, chlorogenic acid, and polyphenols are potent antioxidants and contribute to the radical-scavenging activity of vegetables. The decrease in this activity paralleled the decrease in contents of total phenol, chlorogenic acid, and ascorbic acid in burdock and lettuce (Figs. 1–4). In the case of broccoli, however, the radical-scavenging activity was reduced by a decrease in ascorbic acid (Figs. 1 and 4). Heating inactivated polyphenol oxidase and ascorbate oxidase, which decrease in total phenol, chlorogenic acid and ascorbic acid, and thus broccoli retained its original radical-scavenging activity. An investigation of radical-scavenging activity of heated vegetables was reported previously. Maeda *et al*. (1992) stated that an increase in the radical-scavenging activity of vegetables after boiling might be due to the thermal destruction of vegetable cell walls and subcellular compartments which liberates more components, and/or to thermal chemical reaction which produces more

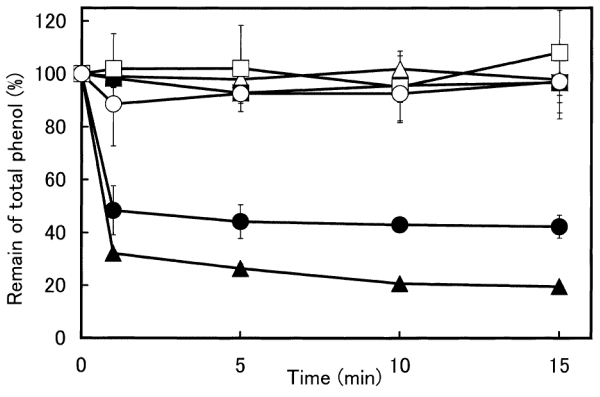

Fig. 2. Effects of heating on the total phenol content during cooking process of vegetables. ▲, burdock; ●, lettuce; ■, broccoli; △, heated burdock; ○, heated lettuce; □, heated broccoli.

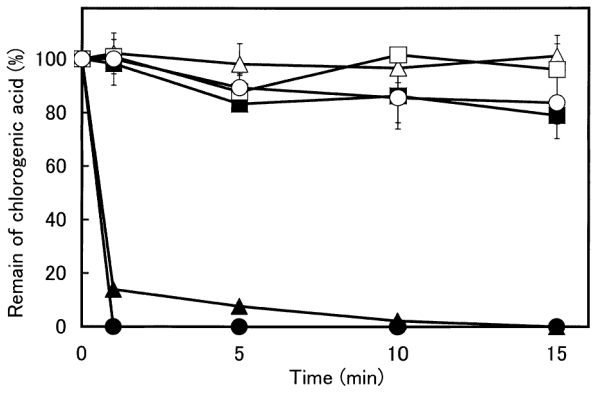

Fig. 3. Effects of heating on the chlorogenic acid content during cooking process of vegetables. ▲, burdock; ●, lettuce; ■, broccoli; △, heated burdock; ○, heated lettuce; □, heated broccoli.

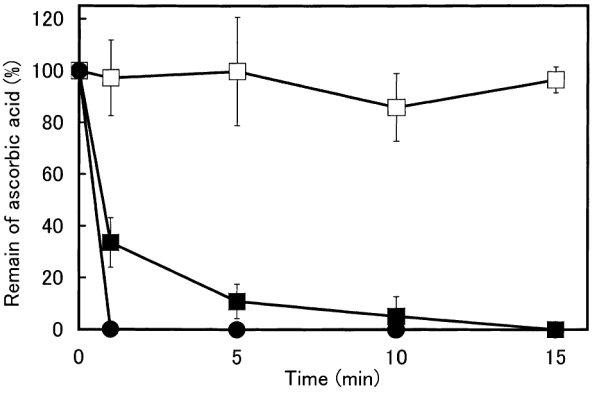

Fig. 4. Effects of heating on the ascorbic acid content during cooking process of vegetables.●, lettuce; ■, broccoli; □, heated broccoli.

potent radical-scavenging antioxidants. Based on our findings, however, we suggest that suppression of the oxidation of antioxidants by thermal inactivation of polyphenol oxidase and ascorbate oxidase resulted in retaining the radical-scavenging activity in heated vegetables.

Conclusions

Burdock and lettuce had high polyphenol oxidase activity, whereas broccoli had the highest ascorbate oxidase activity. In the case of burdock and lettuce, the radical-scavenging activity and the total phenol and chlorogenic acid contents decreased drastically during processing after the first minute. The decrease in radical-scavenging activity paralleled the decrease of chlorogenic acid and total phenol content. In broccoli, however, small decreases in radical-scavenging activity and a stable total phenol and chlorogenic acid content were observed. Thus, the decrease in radical-scavenging activity in this vegetable is dependent on the oxidation of ascorbic acid by ascorbate oxidase. Interestingly, no decrease in radical-scavenging activity, total phenol, chlorogenic acid, or ascorbic acid content were observed when enzymes were inactivated by heating. These findings show that the heating of vegetables is a useful process to prevent the loss of radical-scavenging activity and antioxidants, such as polyphenols and ascorbic acid.

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