Evaluation of Three Antioxidants and their Identification and Radical Scavenging Activities in Edible Chrysanthemums

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Summary

The antioxidant and radical scavenging activities of four edible chrysanthemum cultivars were assayed. Both components showed marked activities, but the difference among them was not significant. The active compounds were isolated by gel permeation chromatography and HPLC, and their antioxidant activity was characterized by a coulometric detection system. Chlorogenic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid were identified as antioxidants in edible chrysanthemums.

Key Words: antioxidant activity, chlorogenic acid, dicaffeoylquinic acids, edible chrysanthemum, radical scavenging activity.

Introduction

Edible chrysanthemum cultivated mainly in the Tohoku district of Japan is a traditionally eaten flower. Its flavorful, colorful petals are long known to contain components that are good for health. Therefore, they have been used not only for liquor “Kikuka-shu” and tea “Kikubana-cha”, but also as a Chinese medicine called “Kikuka”. It has been reported that the edible chrysanthemum contains more ascorbic acid than does the ornamental chrysanthemum (Kagawa, 1988). Recently, other functions of chrysanthemum, anti-allergic activity (Toyoda et al., 1997; Kuppusamy, 1990), anti-inflammatory effect (Akihisa et al., 1996), tumor inhibition (Yasukawa et al., 1996), lens aldose reductase inhibition (Terashima et al., 1991), effects on rat hepatic lipid peroxidation, and activities of aminopyrine N-demethylase and aniline hydroxylase in vivo (Mayanagi et al., 1992), and anti-HIV activity, have been reported (Hu et al., 1994).

Duh and Yen (1997) demonstrated that the chrysanthemum flower, ‘Hang Chu’, had marked antioxidant activity. Tateyama et al (1997a, b) reported that petal extracts of edible chrysanthemum show antioxidant and effective radical scavenging activities, and that their activities correlate with polyphenol and flavonol contents. However, edible chrysanthemum cultivars, tested in their study were indistinct, and the predominant phenolic compounds were not identified. In this study, we examined antioxidant and radical scavenging activities in four edible chrysanthemum cultivars and identified three polyphenolic antioxidants.

Materials and Methods

Materials

Four edible chrysanthemum cultivars, ‘Mottenohoka’, ‘Ki-Mottenohoka’, ‘Shirono’, and ‘Kogane’ were collected in Yamagata Prefecture and cultivated at National Agricultural Research Center for Tohoku Region (Fukushima). The flowers were harvested at the peak of anthesis in 1997. Garland chrysanthemum was purchased from a local supermarket. Xanthine oxidase was purchased from Boehringer Mannheim. Caffeic acid, chlorogenic acid, ferulic acid, and coumaric acid were purchased from Sigma (St. Louis, MO), and 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, which were purified from coffee beans as described below, were used as authentic standards.

Antioxidant assay

Two g samples were homogenized with a Polytron and extracted with 10 ml of 80% methanol. The methanol extract was centrifuged at 10,000 rpm for 10 min and filtered through Advantec No. 2 filter paper. The final volume was adjusted to 20 ml.

The antioxidant activity was assayed by the β-carotene degradation method according to Tsuchida et al. (1994). After mixing 0.5 ml of β-carotene (1 mg·ml\(^{-1}\) chloroform), 0.2 ml of linoleic acid (100 mg·ml\(^{-1}\) chloroform) and 1.0 ml of Tween 40 (200 mg·ml\(^{-1}\) chloroform), chloroform was removed under a stream of...
nitrogen. The mixture was dissolved in 100 ml of 20 mM sodium phosphate buffer (pH 6.8) and a 5-ml aliquot was transferred to a tube containing 0.05 ml of the sample. The tube was incubated in a water bath at 50 °C and the rate of β-carotene discoloration coupled with autoxidation of linoleic acid was measured as absorbance at 470 nm on a UV–VIS spectrophotometer (Hitachi). The antioxidant activity was expressed as inhibition rate (Igarashi et al., 1993), based on the decrease in absorbance during 60 min: Inhibition rate (%) = 100 × (A0 - A) / A0 where A0 is the decrease in absorbance for control, A is that for reaction mixture containing the sample.

Radical scavenging activity

Radical scavenging activity was assayed by the nitrite -kit method, according to Oyanagi (1987). After mixing 0.4 ml of 0.5 mM hypoxanthine, 0.1 ml of 20 mM hydroxylamine -HCl, 0.1 ml of 2 mg -ml⁻¹ hydroxylamine -NO sulfonic acid, 0.8 ml of buffer (32.5 mM KH₂PO₄, 17.5 mM sodium borate, 0.25 mM EDTA -2Na, pH 8.2), a 0.1 ml aliquot and 0.1 ml of xanthine oxidase(4.6 × 10⁻⁵U • ml⁻¹), the mixture was incubated at 37 °C for 30 min. Four milliliters of the color development solution (30 μM naphthylethylene diamine -HCl, 3 mM sulfanilic acid, 25% acetic acid) was added to the mixture which was left to stand at room temperature for 60 min. The pink color development, coupled with superoxide generation, was measured spectrophotometrically as absorbance at 550 nm. The results were calculated as the radical scavenging rate (%), using 100 × (A0 - A) / A0 where A0 is the absorbance of the control; A is that of the sample.

Isolation by chromatography

Petals of edible chrysanthemum ‘Mottenohoka’ were homogenized as above and the crude extract concentrated with a rotary evaporator and then freeze-dried. One gram of freeze-dried extract, dissolved in 20 ml of 0.2% formic acid, was subjected to gel permeation chromatography on a 40 × 450 mm column (Toyopearl HW-40c; Tosoh, Tokyo) with a linear gradient of 30-100% methanol in water containing 0.2% formic acid. Each fraction (15 ml) was collected, and the absorbances at 280 nm, 320 nm and 360 nm measured on a UV–VIS spectrophotometer. The antioxidant activity of each peak was assayed by the β-carotene degradation method.

The active fractions were further purified by high performance liquid chromatography (HPLC; Tosoh) on a 25 × 250 mm column (LiChrosorb RT-18, Merck). The samples were eluted with the linear gradient of 42-75% methanol in water containing 5% formic acid at a flow rate of 5.0 ml • min⁻¹. The UV detector was set at 315 nm.

HPLC coupled with coulometric detection of electroactive components

The HPLC system consisted of a Shimadzu LC-10AD pump, SIL-10AXL autoinjector and SPD-M10AV photodiode array detector. The data were processed by Shimadzu CLASS-LC10 system, equipped with a column (4 × 250 mm, LiChrospher 100RP-18, Merck) in a Shimadzu CTO-10A thermostat-controlled system, and operated at 40 °C. The following solvents were used: solvent A, 10 mM aqueous phosphoric acid; solvent B, 100% methanol containing 10 mM phosphoric acid. Separations were performed with a linear gradient of 10–70% solvent B over a period of 40 min at a flow rate of 1.0 ml • min⁻¹. Electroactive components were detected by HPLC, coupled with an ESA coulometric detection system (ESA Inc., Chelmsford, MA), at the sensor potential of 100 and 400 mV. A sample volume of 10 μl was used for injection.

Identification of active antioxidants

The UV absorption spectra were recorded on a SPD-M10AV photodiode array detector. The active compounds, purified by HPLC, were dissolved in 1 N HCl and hydrolyzed in boiling water for 60 min. The active compounds and their hydrolysates were detected by HPLC, and their retention times were compared with those of authentic compounds.

HR-ESI-MS was measured with a ThermoQuest LCQ mass spectrometer and a Bruker Daltonics APEX II 70e mass spectrometer (Billerica, MA, USA). Samples were dissolved in methanol: H₂O: acetic acid (49:49:2, v/v) and delivered to the ESI source by a mechanical syringe pump. The ²H NMR data at 600 MHz, the ¹³C NMR data at 151 MHz, and the ¹H-¹H COSY data were recorded on a JNM-A600 spectrometer (JEOL Ltd., Akishima, Japan) at room temperature in acetone-d₅.

Results and Discussion

Antioxidant activity and radical scavenging activity

Although garland chrysanthemum is known to show high antioxidant activity (Tsushida et al., 1994), the inhibition rates of the methanol extracts from 4 edible chrysanthemum cultivars were higher (Fig. 1). There were no significant differences in antioxidant activity among the four cultivars. The 'Mottenohoka' flower is pink, whereas the others are yellow. Shirone flowers in the summer, the others bloom in autumn. The flowering characteristics of four cultivars did not correlate with their antioxidant activity. The radical scavenging activity of the four edible chrysanthemum cultivars correlated with their antioxidant activity (Fig. 1, 2). Duh and Yen (1997) reported that an aqueous extract from the flower of *Chrysanthemum morifolium* Ramat also showed marked
antioxidant activity in a liposome model system. When Tateyama et al. (1997a,b) compared antioxidant and radical scavenging activities of petals from different species, they found that the activities of Compositae (chrysanthemum family) were lower than those of the others. However, most petals that they tested, such as the rose, were generally not edible. It is not practical to compare the activities of an edible chrysanthemum with inedible ones.

Separation of active compounds

Three major peaks, P1, P2, and P3, were detected at λ max of 280 nm, 320 nm, 360 nm by Toyopearl gel permeation chromatography (Fig. 3). The antioxidant activities of P3 and P1 (Fig. 4.) were significantly high; their inhibition rates were 28% and 5%, respectively. P2 had little effect on linoleic acid oxidation. When P1 and P3 were purified further by HPLC, only one peak appeared in the HPLC chromatogram of P1; its yield was 1.2 mg per g of freeze-dried crude extract. Two peaks, P3-1 and P3-2, which separated from P3, yielded 2.9 mg and 1.3 mg, respectively. The fractions, P1, P3-1, and P3-2, had nearly identical peak heights at retention times of 15.6, 25.8, and 28.6 min in HPLC chromatograms of methanol extract (Fig. 5A).

Identification of three active antioxidants

The UV-VIS spectra, using a photodiode array detector, showed that all three fractions had their λ max at 325 nm. Acid hydrolysis of these fractions generated a common peak with a retention time of 16.8 min in the HPLC chromatogram. The retention time was identical.
with that of caffeic acid, indicating that these fractions contain caffeoylquinic acid. Tentative identification of these fractions was achieved by comparing the fractions with chlorogenic acid and quinic acid derivatives purified from coffee beans. These quinic acid derivatives gave well-separated peaks at retention times of 15.6, 25.1, 25.8, and 28.6 min; the elution order corresponded to chlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. Co-chromatography of the quinic acid derivatives gave retention times similar to P1, P3-1, and P3-2, and identical with those of chlorogenic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, respectively. The molecular formulas of P1, P3-1, and P3-2 were verified by high-resolution mass measurement as follows: P1: m/z (MH+): calcd. for C_{18}H_{19}O_{9}, 355.10236; found, 355.10009. P3-1: m/z (MH+): calcd. for C_{26}H_{25}O_{12}, 517.13405; found, 517.13224. P3-2: m/z (MH+): calcd. for C_{26}H_{25}O_{12}, 517.13405; found, 517.13498. The structures of P1 and P3-1 were identified as chlorogenic acid (P1) and 3,5-dicaffeoylquinic acid (P3-1), based on the agreements of 13C NMR data with commercial chlorogenic acid and reference data (Chuda, et al., 1996), respectively. The structure of P3-2 was identified as 4,5-dicaffeoylquinic acid based on 1H, 13C NMR, and 1H-1H COSY analyses.

An HPLC procedure, utilizing reversed-phase chromatography, coupled with a coulometric detection system which was developed to characterize overall antioxidant status in vegetables can be used to identify multi-antioxidants, including phenolic acids and flavonoids. A significant positive linear correlation was found in vegetable extracts between the total antioxidant activities determined by oxygen-radical absorbance-capacity assay and the electrochemical data generated from the coulometric detectors (Guo et al., 1997). The HPLC chromatogram of the methanol extract from edible chrysanthemum, detected with a coulometric detection system (Fig. 5B), reveal 3 large peaks identified as P1, P3-1, and P3-2 from the retention times of the isolated fractions. No attempt was made to identify the structures corresponding to the smaller peaks. The peak with a retention time of 33.3 min, which is the tallest in Fig. 5A, was not detected at the sensor potential of 100 mV. The compound at this peak is not an active antioxidant or a radical scavenger.

These results show that chlorogenic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid are main antioxidants in edible chrysanthemum, and that 3,5-dicaffeoylquinic acid is the predominant contributor to the antioxidant activity. However, a synergistic effect of these antioxidants or the other compounds may exist.
Fig. 5. HPLC chromatograms of methanol extract from edible chrysanthemum 'Mottenohoka'. The elute was monitored with (A) photodiode array detector (315 nm) and (B) coulometric detection system (100 mV).

Fig. 6. Chemical structures of the antioxidants isolated from edible chrysanthemum.

P1: \( R_1, R_2 = H, \ R_3 = \text{caffeoyl} \)

P3-1: \( R_1, R_3 = \text{caffeoyl}, \ R_2 = H \)

P3-2: \( R_1 = H, \ R_2, R_3 = \text{caffeoyl} \)

because the antioxidant activities of P1 and P3 are significantly lower than that of the crude extract (Fig. 1, 4). Chlorogenic acid and dicafeoylquinic acids are well-known coffee components and are found in a wide range of fruits and vegetables (Clifford, 1999). Chlorogenic acid and 3,5-dicafeoylquinic acid possess antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging system and the superoxide anion-mediated linoleic acid peroxidation system; they inhibit haemolysis and peroxidation of mouse erythrocytes (Ohnishi et al., 1994). In a low density lipoprotein (LDL) oxidation model, chlorogenic acid efficiently blocked LDL oxidation initiated by ferrylmyoglobin (Laranjinha et al., 1996); it was effective as a peroxyl radical scavenger (Castelluccio et al., 1995). Their results support our conclusion. In addition, these compounds have multiple biological and pharmacological properties, such as antimutagenic activity (Yamada and Tomita, 1996), hepatoprotective activity (Kapil et al., 1995, Basnet et al., 1996), protective effects against
chromosomal damage (Abraham et al., 1993), suppression of the N-nitrosation (Kono et al., 1995), inhibition effect of melanogenesis (Shimozono et al., 1996) and anticarcinogenesis (Mori et al., 1986, Huang et al., 1988, Tanaka et al., 1993). Thus, these functions are also expected to be performed by constituents of edible chrysanthemums.

Some phenolic compounds exist in petals of edible chrysanthemum 'Mottenohoka' other than those antioxidants isolated in the study (Fig. 5A). The HPLC-PDA analysis of P2 hydrolysate indicates that the fraction contains flavonoids, such as apigenin and luteolin in abundance. Although their antioxidant activities are not so high, they may have a synergistic effect with the isolated antioxidants and other beneficial physiological functions. The isolation of these flavonoids is in progress in our laboratory where 17 edible chrysanthemum cultivars are preserved. We wish to seek their antioxidant levels and activities.

Literature Cited


食用ギクの抗酸化性, ラジカル消去活性の評価および抗酸化成分の同定

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摘要

食用ギク4品種の抗酸化性およびラジカル消去活性を評価した。これらの花弁のメタノール抽出物はいずれも強い活性を示したが、品種間の差はみられなかった。ゲル通過クロマトグラフィーおよび高速液体クロマトグラフィーによって、活性を有する物質を単離するとともに、クーロメク検出器を用いてその抗酸化性を調査した。その結果、食用ギクの抗酸化性に関わる主な物質は、クロロゲン酸、3,5-ジカフェオイルキ酸、4,5-ジカフェオイルキ酸であることが明らかになった。