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Antimutagenicity of Mono-, Di-, and Tricaffeoylquinic Acid Derivatives Isolated from Sweetpotato (*Ipomoea batatas* L.) Leaf

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The caffeoylquinic acid derivatives, 3-mono-*O*-caffeoylquinic acid (chlorogenic acid, ChA), 3,4-di-*O*-caffeoylquinic acid (3,4-diCQA), 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA), 4,5-di-*O*-caffeoylquinic acid (4,5-diCQA) and 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-triCQA), and caffeic acid (CA) were isolated from the sweetpotato (*Ipomoea batatas* L.) leaf. We examined the antimutagenicity of these caffeoylquinic acid compounds to promote new uses of the sweetpotato leaf. These caffeoylquinic acid derivatives effectively inhibited the reverse mutation induced by Trp-P-1 on *Salmonella typhimurium* TA 98. The antimutagenicity of these derivatives was 3,4,5-triCQA > 3,4-diCQA = 3,5-diCQA = 4,5-diCQA > ChA in this order. There was no difference in the antimutagenicity of all dicaffeoylquinic acid derivatives. A comparison of the activities and structures of these compounds suggested that the number of caffeoyl groups bound to quinic acid played a role in the antimutagenicity of the caffeoylquinic acid derivatives. The sweetpotato leaves contained distinctive polyphenolic components with a high content of mono-, di-, and tricaffeoylquinic acid derivatives and could be a source of physiological functions.

Key words: antimutagenicity; caffeoylquinic acid derivative; sweetpotato leaf; polyphenol

Agriculturists and nutritionists faced with the problem of feeding the world's hungry are becoming increasingly interested in previously neglected tropical green leafy vegetables such as sweetpotato greens.[1)] Villareal *et al*.[2)] have reported that the sweetpotato leaf is considered to be tougher than other leafy vegetables in the tropics. Our previous report has revealed that the nutritive components of the sweetpotato leaf were comparable to those of commercial leafy vegetables.[3)] Furthermore, sweetpotato tops can be continuously harvested over many months, not just once like many other commercial vegetables.[3)] Repetitive harvesting can thus solve the problem of the low yield of the sweetpotato leaf. It therefore seems desirable to utilize the leaves as the raw material for noodles, bread, drinks, and confectionery rather than just as a fresh vegetable. We have recognized that we must identify physiological functions that are useful for maintaining and improving health and for promoting the sweetpotato leaf, and we also believe that we can utilize the sweetpotato leaf as a functional food material.

We have recently reported the sweetpotato leaf to be an excellent source of polyphenolic compounds.[4)] Phenolic compounds are ubiquitous bioactive compounds and a diverse group of secondary metabolites universally present in higher plants.[5)] Polyphenolics have attracted special attention because they can protect the human body from the oxidative stress which may cause many diseases including cancer, aging and cardiovascular problems.[6–15)] However, little is known about the relationship between the physiological function of the caffeoyl derivatives of the sweetpotato leaf and their chemical structures.

The recent development of screening methods for environmental carcinogens by determining their mutagenicity has enabled various types of mutagen and carcinogen to be detected and identified in daily foods.[16)] It is now known that various types of inhibitors that act against mutagens and carcinogens are present in our daily food, and that they play an important role in reducing the risks of mutagenesis and carcinogenesis.[17)]

We describe in the present paper the antimutagenicity of individual caffeoylquinic acid derivatives iso-

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Abbreviations: CA, caffeic acid; ChA, chlorogenic acid (3-mono-*O*-caffeoylquinic acid); 3,4-diCQA, 3,4-di-*O*-caffeoylquinic acid; 3,5-diCQA, 3,5-di-*O*-caffeoylquinic acid; 4,5-diCQA, 4,5-di-*O*-caffeoylquinic acid; 3,4,5-triCQA, 3,4,5-tri-*O*-caffeoylquinic acid; Trp-P-1,3-amino-1,4-dimethyl-5*H*-pyrido-(4,3-*b*)indol

lated from the sweetpotato leaf and discuss the relationship between this antimutagenicity and the chemical structure. Clarification of the relationship between the chemical structure and physiological functions will enable new varieties of sweetpotato to be selected with desired physiological functions.

Materials and Methods

Sweetpotato leaf materials. The sweetpotato varieties raised for use as leafy vegetables in the National Agricultural Research Center for Kyushu Okinawa Region (KONARC), Japan, were used for the present study. Ten varieties were cultivated from March 15, 2001, under the same conditions in an experimental field in Miyakonojo (Japan). The tops were harvested on July 22, 2001, and separated into three portions, the leaf, the petiole, and the stem. Each sample was washed, lyophilized, powdered, and kept at 5°C until needed.

Chemicals and bacteria. Trp-P-1 and caffeic acid (CA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Chlorogenic acid (ChA) was purchased from Sigma Chemical (St. Louis, MO, U.S.A.). S-9 fractions prepared from rat liver that had been pretreated with phenobarbital and 5,6-benzoflavone, and cofactor I were products of Oriental Yeast Co. (Tokyo, Japan). All other chemicals used were of special grade. Strain TA 98 of *Salmonella typhimurium* was supplied by the Institute for Fermentation in Osaka (IFO), Japan.

Extraction and measurement of phenolics. The lyophilized sweetpotato powder was vigorously mixed with 10 times its equivalent volume of 80% ethanol. The mixture was boiled for 5 min under a hood and centrifuged at $5000 \times g$ for 10 min. The residue was mixed with additional 80% ethanol and boiled for 10 min to re-extract the phenolics, before being centrifuged under the same conditions. The extracts were combined and made up to 10 ml, this combined extract being used for measuring total phenolics. Total polyphenols were determined according to the Folin-Ciocalteu method with a slight modification.[18] The alcohol extract was diluted to obtain an absorbance reading within the range of the standards (800 to 40 μg of ChA/ml). The absorbance in the microplate wells was measured at 600 nm with a dual-wavelength flying-spot scanning densitometer (Shimadzu Co., Kyoto, Japan) fitted with a microplate system, the results being expressed as g of ChA/100 g of powder.

Quantification of phenolic acids by HPLC. The lyophilized sweetpotato leaf powder (50 mg) was vigorously mixed with 4 ml of ethanol in a centrifuge tube fitted with a cap. The mixture was boiled for 5 min under a hood and centrifuged at $3000 \times g$ for 10 min. The supernatant was filtered through a cellulose acetate membrane (0.20 μm, Advantec, Tokyo, Japan), and the filtrate used as a sample. A 5-μl portion of the filtrate was injected into an HPLC system consisting of two LC-10AT pumps, an SIL-10AXL autoinjector, a CTO-10AC column oven and an SPD-M10AVP UV-VIS photodiode array (Shimadzu Co., Kyoto, Japan). A YMC-Pack ODS-AM AM-302 column (4.6 mm ID × 150 mm, 5 μm particles; YMC, Kyoto, Japan) was used. The temperature was set to 40°C. The mobile phase consisted of water containing 0.2% (v/v) formic acid (A) and methanol (B). Elution was performed with 2%B from 0 to 15 min, a linear gradient of 2% to 45% of B from 15 to 50 min, and 45%B from 50 to 65 min. The flow rate was 1 ml/min. Each polyphenol was compared with an authentic sample.

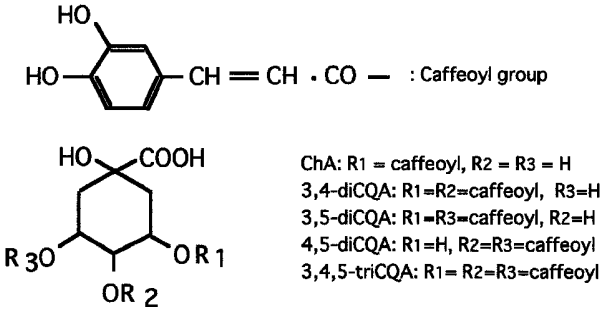

Fig. 1. Chemical Structures of Caffeic Acid and Caffeoylquinic Acid Derivatives Isolated from cv. K66Mu72-2 Leaf.

Isolation of sweetpotato leaf polyphenolics. The dried leaves of sweetpotato (150 g) were extracted with MeOH (2 l, two times) at room temperature. The extract (17 g) was partitioned between benzene and water. The aqueous layer (8 g) was chromatographed on MCI CHP20P gel (about 50 ml bed volume; Mitsubishi Chemical Co., Tokyo, Japan), eluting with water, 20%MeOH, 40%MeOH, 60% MeOH, 80%MeOH, and 100%MeOH in succession. The 40% and 60% eluates were chromatographed in an ODS colomn (30–50 μm, Fuji Silisia Ltd., Nagoya, Japan; 25 mm ID × 140 mm; 20% to 70% MeOH) to yield CA (15 mg), ChA (400 mg), 3,4-diCQA (2 mg), 3,5-diCQA (60 mg), 4,5-diCQA (21 mg), and 3,4,5-triCQA (2 mg). Each compound was identified by its ^{1}H- and ^{13}C-NMR and FAB-MS data. The NMR spectra were taken with a Jeol A-500, and FAB-MS data with a Jeol JMS-DX303HF spectrometer. Purified (>97%) 3,4-diCQA, 3,5-diCQA, 4,5-diCQA and 3,4,5-triCQA were used as standards for the HPLC analysis. The chemical structures of the caffeoylquinic acid derivatives are shown in Fig. 1.

Assay for antimutagenicity. The antimutagenic ac-

Table 1. Total Polyphenol Content of Different Varieties of Sweetpotato Leaf

Variety	Total polyphenolic content (g/100 g of powder)
K66Mu72-2	4.52
Tsurusengan	2.81
Kyukei 123	6.81
S106-190	5.63
Kyukei 84048-28	4.49
Kyukei 7181-32	2.99
Kyushu No. 116	9.24
Kyukei 58	6.89
Elegant Summer	6.48
Simon No. 1	6.67

tivity was evaluated for *Salmonella typhimurium* TA 98 by using the Trp-P-1 mutagen.[14] An S-9 mix was prepared from S-9 and cofactor I according to the manufacturer's recommendation. For the inhibition test, 0.1 ml of the mutagen, 0.1 ml of the DMSO-dissolved polyphenolic solution, and 0.5 ml of the S-9 mix were simultaneously incubated with 0.1 ml of a bacterial suspension at 37°C for 20 min, before being poured on to minimal-glucose agar plates with 2 ml of soft agar. The colony number on each plate was counted after 48 hr of cultivation at 37°C.

Results

Total polyphenolic content

Table 1 shows the total polyphenolic content of the leaf from the 10 varieties raised as leafy vegetables by KONARC. The content varied from 2.81 g/100 g of powder to 9.24 g/100 g of powder. The polyphenol content of the Elegant Summer variety, which was developed for edible use of the petiole, was 6.48 g/100 g of powder, and that of Simon No. 1, which was developed for medical purposes, was 6.67 g/100 g of powder. Cv. K66Mu72-2 had a polyphenolic content of 4.52 g/100 g of powder and was selected for isolating the caffeoylquinic acid derivatives, since both the yield and taste were superior to those of the other varieties.

Isolation of the caffeoylquinic acid derivatives

CA and five caffeoylquinic acid derivatives (ChA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, and 3,4,5-triCQA) were isolated from the leaves of cv. K66Mu72-2. The HPLC chromatographic pattern is shown in Fig. 2. The individual content of polyphenolic compounds in the cv. K66Mu72-2 leaf was in the following order: 3,5-diCQA (91 mg/100 g of powder) > 4,5-diCQA (49 mg/100 g of powder) > ChA (10.3 mg/100 g of powder) > 3,4-diCQA (9 mg/100 g of powder) > 3,4,5-triCQA (4 mg/100 g of powder) > CA (2 mg/100 g of powder) (Table 2). The average ratios of the polyphenolic components from cv. K66Mu72-2 were 3,5-diCQA (49%), 4,5-diCQA (26%), ChA (17%) and 3,4-diCQA (5%), and minor components 3,4,5-triCQA (2.0%) and CA (1.3%). The average ratios of polyphenolic components from 20 genotypes were 3,5-diCQA (49%), 4,5-diCQA (23%), ChA (13%), and 3,4-diCQA (10%); the minor components were 3,4,5-triCQA (2.7%) and CA (1.1%). The ratios of polyphenolic components from the cv. K66Mu72-2 leaf were almost the same as those from the 20 genotype leaves. This result suggests that the main polyphenolic compound in sweetpotato leaves is 3,5-diCQA, followed by 4,5-diCQA. All the HPLC profiles of the other varieties also showed peaks at the same retention times, there being no quantitative difference between the varieties.[4] Therefore, the six quoted caffeic acid derivatives universally existed in the sweetpotato leaf. Each caffeoylquinic acid derivative was purified and used for the subsequent experiments.

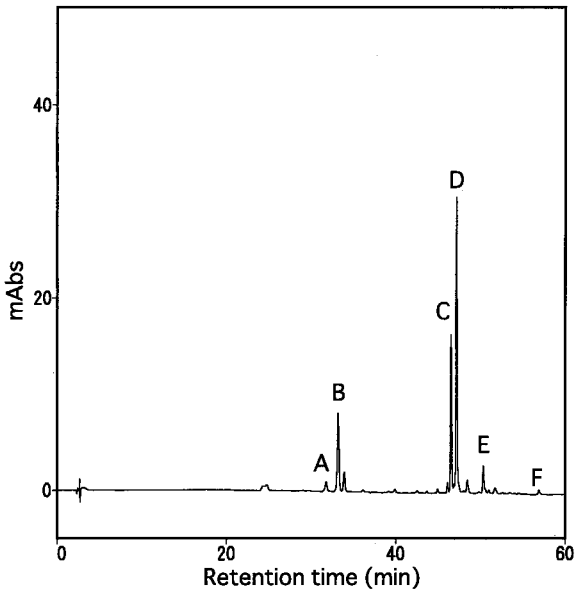

Fig. 2. HPLC Chromatogram of Caffeic Acid and Caffeoylquinic Acid Derivatives in an 80% Ethanol Extract from cv. K66Mu72-2 Leaf.

A, caffeic acid (Rt = 32.27 min); B, chlorogenic acid (Rt = 33.42 min); C, 4,5-di-*O*-caffeoylquinic acid (Rt = 46.72 min); D, 3,5-di-*O*-caffeoylquinic acid (Rt = 47.34 min); E, 3,4-di-*O*-caffeoylquinic acid (Rt = 50.55 min); F, 3,4,5-tri-*O*-caffeoylquinic acid (Rt = 57.25 min).

Antimutagenicity of the caffeoylquinic acid derivatives

The effect of each caffeoylquinic acid derivative on the reverse mutation induced by Trp-P-1 is shown in Table 3. ChA inhibited the reverse mutation by 29–41% in a dose range of 0.14–0.57 mM, while 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA respectively inhibited the reverse mutation by 39–59%, 25–59%, and 32–61%. The 3,4,5-triCQA derivative inhibited the reverse mutation by 46–84% in the same dose range of 0.14–0.57 mM. All compounds tested

Table 2. Content of Caffeic Acid and Caffeoyl Derivatives in cv. K66Mu72-2 Leaf

	Caffeoyl derivatives (mg/100 g of lyophilized powder)[a]					
	CA	ChA	3,4-diCQA	3,5-diCQA	4,5-diCQA	3,4,5-triCQA
K66Mu72-2	2(1.3)	31(16.8)	9(4.7)	91(48.9)	49(26.3)	4(2.0)
Ratio (%)[b]	1.1	13.1	10.3	49.4	23.4	2.7

[a] Values in parentheses are the percentage of caffeic acid derivatives in cv. K66Mu72-2 leaf.
[b] Values are average for caffeic acid derivatives in 20 genotype leaves.

Table 3. Effect of Caffeoylquinic Acid Derivatives from Sweetpotato Leaves on the Mutagenicity of Trp-P-1 against *Salmonella typhimurium* Ta 98[a]

Sample	Dose (mM)	His^+ revertants (/plate[b])	Inhibition (%)
ChA	0.14	369± 5	29
	0.29	335±23	35
	0.57	307±15	41
3,4-diCQA	0.14	317± 6	39
	0.29	267± 6	48
	0.57	211±13	59
3,5-diCQA	0.14	391±19	25
	0.29	283±13	45
	0.57	213±20	59
4,5-diCQA	0.14	350±14	32
	0.29	241±27	54
	0.57	200± 9	61
3,4,5-triCQA	0.14	281±27	46
	0.29	137±22	74
	0.57	85± 6	84

[a] Trp-P-1 was added at a dose of 0.075 μg/plate. The mutagenicity was tested with S-9 mix.
[b] Each value represents the mean±S.D. of triplicate plates. The values shown have had the spontaneous mutation frequency subtracted. The His^+ revertant values for the controls of the caffeoylquinic acid derivatives were 518±49/plate.

showed dose-dependent antimutagenicity. No killing effect was apparent, at least in the dose range tested (data not shown). The three dicaffeoylquinic acid derivatives exhibited similar antimutagenic activity at a dose of 0.57 mM. The antimutagenicity of the three dicaffeoylquinic acid derivatives and 3,4,5-triCQA was respectively about 1.5 and 2.0 times higher than that of ChA.

Relationship between the number of caffeoyl groups and the antimutagenicity

We isolated the mono-, di-, and tricaffeoylquinic acid derivatives from the sweetpotato leaf and were thus able to compare the antimutagenicity and structure of those derivatives. Figure 3 shows the relationship between the antimutagenicity of the caffeoylquinic acid derivatives at the dose of 0.57 mM in Table 3 and the number of caffeoyl groups bound to quinic acid. The relationship shows that the antimutagenicity increased linearly ($r=0.994$) with increasing number of caffeoyl groups. The degree of inhibition strengthened at about 20% per caffeoyl group in the derivatives. These results clearly indicate

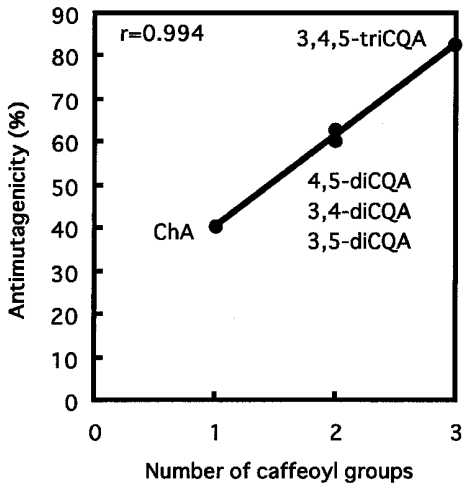

Fig. 3. Relationship between the Antimutagenicity and Number of Caffeoyl Groups Bound to Quinic Acid.

that a number of caffeoyl groups bound to quinic acid was necessary to promote antimutagenicity.

Discussion

The total polyphenolic content of the leaf from each variety tested in this present work varied from 2.81 g/100 g of powder to 9.24 g/100 g of powder (Table 1). Our previous report has indicated that the total polyphenolic content of the sweetpotato root varied from 1.80 g/100 g of powder in Ayamurasaki (purple flesh) to 230–310 mg/100 g of powder in Koganesengan (yellow flesh), Joy White (white flesh), and Sunny Red (orange flesh).[14] These data show that the polyphenolic content of the sweetpotato leaf was much higher than that of the root. Furthermore, we found that the polyphenolic content of the sweetpotato leaf was also much higher than that of commercial vegetables (data not shown). Therefore, the sweetpotato leaf can be utilized as a physiologically functional material.

The caffeoylquinic acid derivatives effectively depressed the reverse mutation induced by Trp-P-1 (Table 3). Our previous report has indicated that the catechol structure played an important role in the strong antimutagenicity of anthocyanin pigments.[19] Furthermore, Yagasaki *et al.*[20] have suggested the possible involvement of the 3,4-dihydroxyl group of

CA in the suppression of hepatoma invasion *in vitro* by an experiment using CA, cinnamic acid, and *p*-coumaric acid (4-hydroxycinnamic acid). ChA, and di- and tricaffeoylquinic acid are esters of quinic acid and one-, two-, and three-caffeic acids. These results suggested that an increasing number of caffeoyl groups promoted antimutagenicity. As we would expect, the caffeoylquinic acid derivatives effectively inhibited the reverse mutation in proportion to the number of caffeoyl groups bound to quinic acid (Fig. 3).

3,5-diCQA and 4,5-diCQA exhibited anti-inflammatory activity *in vitro*, while 3,4,5-triCQA was inactive.[8] ChA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA, which had been extracted from steamed sweetpotato root, suppressed melanogenesis equally well.[10] Yagasaki *et al.*[20] have indicated that ChA, CA, and quinic acid suppressed hepatoma cell invasion without altering the cell proliferation. 3,4,5-triCQA and 4,5-diCQA have been noted to inhibit HIV replication,[21] and 3,5-diCQA to inhibit the histamine secretion induced by concanavalin A plus phosphatidylserine from rat peritoneal mast cells.[22] Murayama *et al.*[23] have recently identified ChA, 3,5-diCQA, and 4,5-diCQA as antioxidants in edible chrysanthemums. Thus, the caffeoylquinic acid derivatives can be expected to protect humans from various kinds of diseases.

The sweetpotato leaf contained CA, ChA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, and 3,4,5-triCQA (Fig. 2). It has also been found that ChA and dicaffeoylquinic acid derivatives were contained in the sweetpotato storage root.[10,24] Although ChA and dicaffeoylquinic acid derivatives have been isolated from various plants as just described, there have been few reports on the 3,4,5-triCQA. The isolation of 3,4,5-triCQA has been reported from *Securidaka longipedunculata* (polygalaceae),[21] *Tessaria integrifolia* and *Mikania cordifolia* (Asteraceae).[8] The results of our present study indicate that the antimutagenicity of 3,4,5-triCQA was more effective than that of the mono- or dicaffeoylquinic acid derivatives. Mahmood *et al.*[21] have reported that 3,4,5-triCQA exhibited greater selective inhibition of HIV replication than 4,5-diCQA, and that CA had only slight anti-HIV activity. These data suggest that 3,4,5-triCQA might have better physiological functions than those of mono- or dicaffeoylquinic acid. The sweetpotato leaf contains 3,4,5-triCQA, suggesting that it is the source of not only mono- and dicaffeoylquinic acid derivatives, but also of 3,4,5-triCQA. We plan to investigate the physiological functions of the caffeoylquinic acid derivatives in the near future.

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