Journal of Health Science, **51**(4) 492–496 (2005)

Antioxidative Activity of 3-*O*-Octanoyl-(+)-Catechin, a Newly Synthesized Catechin, *in Vitro*

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(Received March 28, 2005; Accepted May 7, 2005)

The antioxidative activities of 3-*O*-octanoyl-(+)-catechin were studied *in vitro*. Free radical scavenging activities were tested by spectrophotometrically measuring 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) radical cations, as well as by measuring luminol chemiluminescence induced by peroxyl radicals generated from 2,2′-azinobis(2-amidinopropane) dihydrochloride (AAPH). The radical scavenging activities of the 3-*O*-octanoyl-(+)-catechin for ABTS radical cations and peroxyl radicals were less effective than the original (+)-catechin, but stronger than 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox). Furthermore, 3-*O*-octanoyl-(+)-catechin unlike (+)-catechin did not effectively antagonize the decrease in the intrinsic fluorescence intensity of allophycocyanin induced by AAPH, which was measured as an index of protein damage. Conversely, 3-*O*-octanoyl-(+)-catechin demonstrated more potent antioxidative activity for the linoleic acid peroxidation induced by AAPH than (+)-catechin and Trolox. These findings suggest that the introduction of an octanoyl group at the 3-OH position in a (+)-catechin effectively quench the secondary products of lipid peroxidation without much loss of free radical scavenging activity.

Key words —— 3-*O*-octanoyl-(+)-catechin, antioxidative activity, lipid peroxidation

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INTRODUCTION

Catechins belong to a subclass of flavonoids distributed widely in higher plants. The tea plant *Camellia sinensis* L, which contains catechins as the major constituent group, has been used as a beverage for thousands of years in East Asia. Recently, tea extract, or catechins, have been recognized as health-promoting factors owing to their antimutagenic,[1] antioxidative,[2,3] and antibacterial activity.[4]

It has been reported that several newly synthesized 3-*O*-acyl-(+)-catechins, as well as 3-*O*-acyl-(–)-epigallocatechins, inhibited the activation of the Epstein-Barr virus early antigen more strongly than catechins lacking the 3-*O*-acyl group.[5,6] 3-*O*-Octanoyl-(+)-catechin was one of the most effective derivatives. The evidence suggests that modification of 3-OH group of catechins could enhance the biological functions of the original catechins. Although (+)-catechin, having the basic structure of flavan-3-ol, is a minor component among tea catechins and inferior to other catechin derivatives in terms of biological activities, the modification of the 3-OH of (+)-catechin may contribute to an increase in biological activities. In the present study, we evaluated the antioxidative activity of 3-*O*-octanoyl-(+)-catechin either as a free radical scavenger or as an antioxidant against the oxidation of lipids or proteins *in vitro*.

MATERIALS AND METHODS

Reagents —— 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), 2,2′-azinobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), 3-cyclohexylaminopropanesulfonic acid (CAPS), luminol, and allophycocyanin were obtained from Wako Pure Chemicals, Osaka, Japan. 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) was a product of Sigma-Aldrich, Milwaukee, U.S.A. 3-*O*-Octanoyl-(+)-catechin was synthesized and purified as described in a previous report.[6] Other chemicals were of reagent grade.

ABTS Radical Cation Decolorization Assay —— The radical scavenging activity of antioxidants for ABTS radical cations was measured according to the methods of Re *et al*.[7] A stock solution of ABTS radical cations was prepared one day before the assay by mixing 5 ml of 7 mM ABTS with 1 ml of 14.7 mM potassium persulfate, followed by storage

in a dark at room temperature. The stock solution of ABTS radical cations was diluted with water or ethanol to an absorbance of about 0.70 at 734 nm. The decolorization assay was started by mixing 2 ml of the diluted ABTS solution with 20 μl of a test compound solution in a cuvette. Test compounds were dissolved with 10% dimethylsulfoxide (DMSO). At 4 min after mixing, the absorbance was measured at 734 nm. In a control experiment, a test compound solution was replaced with 10% DMSO, and the radical scavenging activity of the test compound was expressed as a percentage inhibition based on the absorbance.

AAPH-Induced and Luminol-amplified Chemiluminescence Assay —— Luminol-amplified chemiluminescence in the presence of free radical generator AAPH was measured by the method of Kawagoe and Nakagawa.[8] The reaction mixture consisted of 400 μl of 150 mM CAPS buffer (pH 10.0), 40 μl of 0.75 mM luminol, 100 μl of 60 mM AAPH, 30 μl of a test compound solution, and 30 μl of distilled water. AAPH solution was prepared 30 min before the assay. In a control experiment, a test compound solution was replaced with 10%DMSO. The chemiluminescent intensity in relative light units was measured for 10 sec at 15 min after the automatic addition of luminol and AAPH solutions in the luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Garmany). The scavenging activity was expressed as a percentage inhibition based on the chemiluminescent intensity.

Measurement of Lipid Peroxidation Induced by Radical Generators *in Vitro* —— Lipid peroxidation of linoleic acid induced by the addition of AAPH was determined by a modification of the method of Osawa *et al*.[9] In a screw cap vial, 1.0 ml of 2.85% linoleic acid in ethanol, 0.1 ml of each test compound in ethanol, and 4.5 ml of 40 mM phosphate buffer (pH 7.0) was mixed with a Vortex mixer for 2 min.

Lipid peroxidation was started by mixing with 0.1 ml of 570 mM AAPH, a water-soluble radical generator, or 57 mM AMVN, a lipid-soluble radical generator, followed by incubation for 3 hr at 37°C. A 0.1 ml of reaction mixture was diluted with 9.7 ml of 75% ethanol, and mixed with 0.1 ml of 30% ammonium thiocyanate. After 3 min, 0.1 ml of 20 mM ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture, followed by measurement of the absorbance at 500 nm.

AAPH-Induced Oxidation of Fluorescent Protein Allophycocyanin —— The intrinsic fluorescent intensity of allophycocyanin altered by the addition of AAPH was measured according to the method of Nakagawa *et al*.[10] with a slight modification. The reaction mixture consisted of 37.5 nM allophycocyanin, 3 mM AAPH, and a sample solution in 75 mM phosphate buffer (pH 7.0). The fluorescent intensity (excitation 598 nm; emission 651 nm) was measured using a spectrophotofluorometer (Hitachi 650-10M, Tokyo, Japan) 10 min after the addition of AAPH solution.

Statistical Analysis —— All values are expressed as the mean ± S.E. Statistical analyses were performed by one-way analysis of variance (ANOVA). The differences among groups were analyzed by Tukey-Kramer test, and were considered significant at $p < 0.05$.

RESULTS

As an ABTS radical cation vehicle at a high volume in a reaction mixture may affect the solubility of test compounds, we used either water or ethanol for the dilution of the ABTS stock solution. ABTS radical cation scavenging activities of (+)-catechin, 3-*O*-octanoyl-(+)-catechin, and Trolox, a water-soluble α-tocopherol derivative, were tested in a water solution or in an ethanol solution (Fig. 1). These three compounds clearly scavenged radicals in a dose–dependent manner in both vehicles. The radical scavenging activity of 3-*O*-octanoyl-(+)-catechin was slightly weaker than that of (+)-catechin, but much stronger than Trolox.

Figure 2 shows the time-course of the radical scavenging reactions of test compounds at a concentration of 10 μM. The interaction between ABTS radical cations and Trolox finished within 30 sec, and radical scavenging activities were almost equivalent in both vehicles. The reaction of 3-*O*-octanoyl-(+)-catechin or (+)-catechin with ABTS radical cations was rather slow in comparison to Trolox, and reached a plateau at approximately 4 min after the initiation of the reaction. The influence of vehicles on scavenging activity for the two catechins used in the present experiment is evident in Fig. 2. (+)-Catechin, superior to 3-*O*-octanoyl-(+)-catechin, demonstrated much higher scavenging activities in water than in ethanol.

The reactivity of these compounds (0.2 μM) against peroxyl radicals generated from AAPH is shown in Fig. 3. The reaction was carried out in an alkaline aqueous phase (pH 10.0). 3-*O*-Octanoyl-(+)-catechin and (+)-catechin attenuated chemilumi-

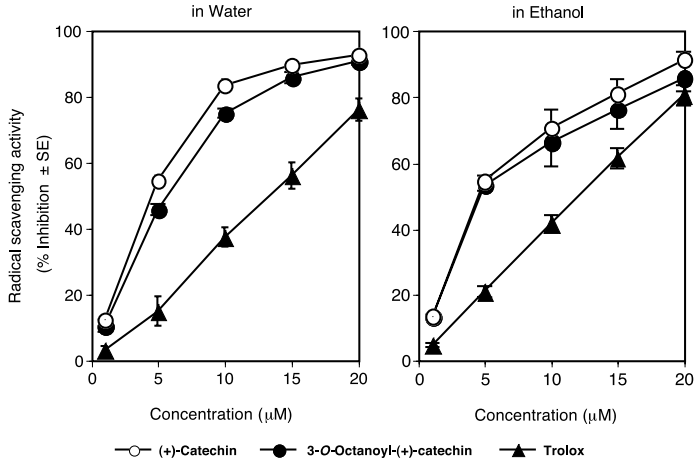

Fig. 1. ABTS Radical Cation Scavenging Activity of Antioxidants in Water or in Ethanol

ABTS radical cation scavenging activity is indicated as % inhibition. Each point represents the mean ± S.E. from 4 separate experiments. Each experiment was carried out in duplicate.

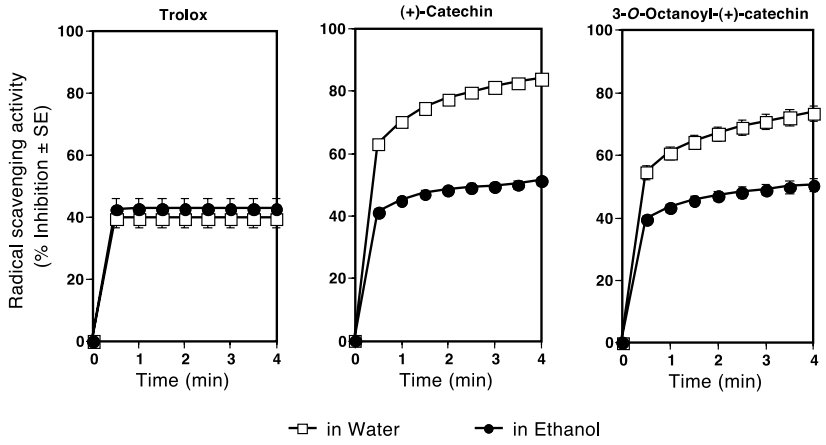

Fig. 2. Time-Course of ABTS Radical Cation Scavenging in the Presence of Antioxidants (10 μM) in Water or Ethanol

The ABTS radical cation scavenging activity is indicated as % inhibition. Each point represents the mean ± S.E. from 3 separate experiments. Each experiment was carried out in duplicate.

nescent intensity by 40.6 and 77.5%, respectively, whereas Trolox reduced it by 7.8%, indicating that these catechins were more reactive for peroxyl radicals than Trolox in an alkaline solution.

Effects of the radical scavengers (10 μM) on the lipid peroxidation of linoleic acid induced by free radical generators were examined (Fig. 4). 3-*O*-Octanoyl-(+)-catechin significantly decreased lipid peroxidation induced by AAPH as compared with (+)-catechin and Trolox. The difference in inhibitory effects on lipid peroxidation between 3-*O*-octanoyl-(+)-catechin and (+)-catechin was prominent when AAPH was replaced with AMVN.

To evaluate the preventive effects of 3-*O*-octanoyl-(+)-catechin against protein damage induced by free radicals, the fluorescence intensity of allophycocyanin was measured in the presence of AAPH. The fluorescence intensity expressed as % of the control was 79.2 ± 0.8 (n = 5), 95.4 ± 4.2 (n = 5), 84.4 ± 2.4 (n = 5) in the presence of AAPH, AAPH plus 10 μM (+)-catechin, and AAPH plus 10 μM 3-*O*-octanoyl-(+)-catechin, respectively. (+)-Catechin, but not 3-*O*-octanoyl-(+)-catechin, significantly antagonized the decrease in fluorescence intensity induced by AAPH ($p < 0.05$).

DISCUSSION

The ABTS radical cation scavenging activity of

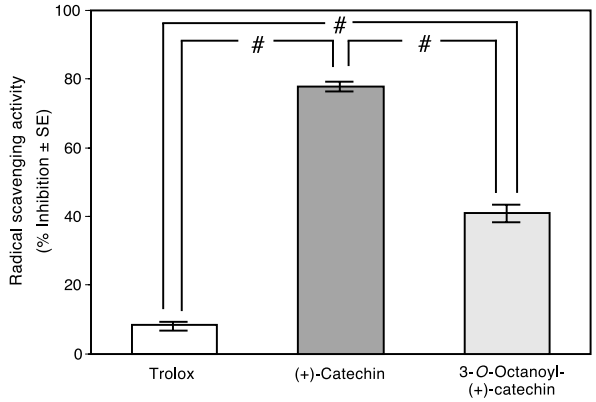

Fig. 3. Inhibitory Effects of Antioxidants (0.2 μM) on Luminol-Amplified Chemiluminescent Intensity Induced by AAPH

The radical scavenging activity is indicated as % inhibition. Each bar represents the mean ± S.E. from 4 separate experiments. Each experiment was carried out in duplicate. #, statistically different at $p < 0.05$.

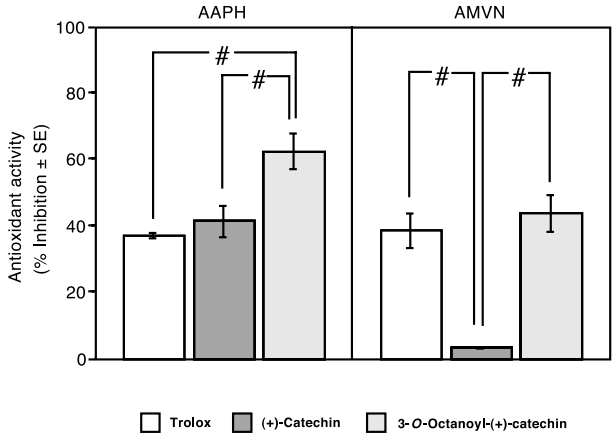

Fig. 4. Inhibitory Effects of Antioxidants on Lipid Peroxidation of Linoleic Acid Induced by AAPH or AMVN

The antioxidant activity is indicated as % inhibition. Each bar represents the mean ± S.E. from 4 separate experiments. Each experiment was carried out in duplicate. #, statistically different at $p < 0.05$.

(+)-catechin in the water solution was about 2.9-fold higher than that of Trolox, based on the 50% inhibition concentration (IC_{50}) of the absorbance of ABTS radical cations calculated from Fig. 1; the IC_{50}s of (+)-catechin and Trolox were 4.6 and 13.6 μM, respectively. Since the results indicate the extent of ABTS radical cations scavenged at the end point of the reaction, the differences in the scavenging activity in the present experiments principally depend on the number of the functional hydroxyl groups participating in the reaction. In fact, the radical scavenging activity of 3-*O*-octanoyl-(+)-catechin, which was esterified at the 3-OH of (+)-catechin with caprylic acid, was slightly weaker than (+)-catechin. Nanjo *et al.*[11] have also reported that the acetylation of the 3-OH of (–)-chatechin slightly reduces the scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radical.

In addition, 3-*O*-octanoyl-(+)-catechin, as well as (+)-catechin, revealed that the ABTS radical cation scavenging activity was more effective in the water solution than in the ethanol solution, whereas the free radical scavenging activity of Trolox was similar in both solutions. The polarity of water may favor these catechins with quenching the radicals in association with the phenolic hydroxyl moieties in catechins.

Many lines of evidence have demonstrated that the phenolic hydroxyl moieties of flavonoids are essential for their antioxidant activities. It has been generally recognized that the ortho-dihydroxyl structure in the B ring and the meta-dihydroxyl structure in the A ring of flavonoids basically contribute to their antioxidant activities, the former being predominant. Moreover, the 4-oxo group conjugated with a 2,3-double bond in combination with the 3-OH in the C ring and the 5-OH in the A ring of flavonoids is important for their maximum radical scavenging potential.[12,13] As (+)-catechin has a saturated C ring without the 4-oxo function, the 3-OH group appears to contribute to antioxidant activities to a lesser extent. The esterification of the 3-OH with the lipophilic moiety in the present study, therefore, did not greatly reduced the radical scavenging activity.

The species of free radicals is likely to be another determinant for radical scavenging activity. In the chemiluminescence assay, peroxyl radicals generated from AAPH were scavenged by the antioxidants in a decreasing order; (+)-catechin > 3-*O*-octanoyl-(+)-catechin > Trolox. The radical scavenging activity of 3-*O*-octanoyl-(+)-catechin was significantly lower than that of (+)-catechin and was different from the ABTS radical cation scavenging activity. Factors other than the radical species are possibly related to the differences. Since the assay was carried out in an alkaline solution (pH 10.0) in the chemiluminescence assay, dissociation of hydroxyl groups may be involved. On this point, the findings of Lemańska *et al.*[14] that the Trolox equivalent antioxidant capacity values for 3-hydroxyflavone, 5-hydroxyflavone, 3′,4′-didihydroxyflavone, and 5,7-dihydroxyflavone increased with in-

creasing pH are suggestive. Further, Nanjo *et al.*[11] have reported that the radical scavenging ability of (+)-catechin increases with increasing pH value.

In contrast to the effects on radical scavenging activities, the inhibitory effect of 3-*O*-octanoyl-(+)-catechin on the lipid peroxidation induced by a hydrophilic radical initiator, AAPH, was significantly stronger than that of (+)-catechin. Furthermore, the attenuation by 3-*O*-octanoyl-(+)-catechin of lipid peroxidation induced by a lipophilic radical initiator, AMVN, was markedly stronger than that of (+)-catechin. Though catechins are fairly lipid soluble by nature, the esterification at the 3-OH of (+)-catechin with the C_8 chain possibly increased the lipid solubility. As this assay estimates principally the secondary products of oxidation of linoleic acid attacked by free radicals, the antioxidant activity would include the quenching of lipid peroxides and lipid peroxyl radicals in addition to free radicals directly produced from radical initiators. On the other hand, the damage to allophycocyanin protein caused by free radicals from AAPH was not blocked by 3-*O*-octanoyl-(+)-catechin, in contrast to (+)-catechin.

Accordingly, the higher affinity of 3-*O*-octanoyl-(+)-catechin for the lipids could give a stronger antioxidant power than (+)-catechin by approaching the vicinity of target molecules more easily. To test the efficacy of 3-*O*-octanoyl-(+)-catechin as an antioxidant in foods or biological systems, further studies are needed.

In conclusion, 3-*O*-octanoyl-(+)-catechin showed a stronger inhibitory effect against lipid peroxidation of linoleic acid than the original (+)-catechin, probably owing to an increase in its accessibility to target oxidants, without greatly reducing free radical scavenging activities.

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