Copper(II) Ions Convert Catechins from Antioxidants to Prooxidants in Protein Carbonyl Formation

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Catechins are naturally occurring polyphenols, which are supposed to have antioxidative effects on foods and living cells. We examined the antioxidative effects of (+)-catechin and 3-*O*-acyl derivatives of (+)-catechin in combination with copper(II) [Cu(II)] ions on the formation of carbonyl groups in bovine serum albumin (BSA) at pH 7.4 by incubating *in vitro* for 90 min at 37°C. In the presence of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), a free radical generator, the formation of carbonyl groups in BSA, which increased time-dependently, was significantly ($p < 0.05$) attenuated by the addition of (+)-catechin and 3-*O*-acyl-(+)-catechins. However, Cu(II) ions, but not Zn(II) ions, blocked the antioxidative effects of (+)-catechin and 3-*O*-octanoyl-(+)-catechin on the oxidative modification of BSA induced by AAPH. On the other hand, Cu(II) ions had no influence on the scavenging activity of (+)-catechin for 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) cation radicals. Furthermore, in the absence of AAPH, coexistence of Cu(II) ions with (+)-catechin greatly accelerated the formation of carbonyl groups in BSA, being dependent on the concentrations of Cu(II) ions and (+)-catechin. These results suggest that Cu(II) ions could convert (+)-catechin from an antioxidant to a prooxidant in protein oxidation.

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INTRODUCTION

Oxidative damage of biological molecules such as lipoproteins, proteins, and nucleotides by free radicals is believed to be associated with the development of atherosclerosis, senescence, cancer, and other disorders in organisms.[1)] Polyphenols, being widely distributed in plants, have antioxidative activities assumed to lead to the prevention of oxidative damage in human.[2,3)] Many human intervention studies have also indicated that the intake of dietary polyphenols increased antioxidant capacity.[4)] On the other hand, polyphenols could act as prooxidants on biological molecules under certain conditions, especially in the presence of oxygen and transition metals.[3,5,6)] Copper(II) [Cu(II)] is a prominent metal in oxidizing polyphenols, although the interactions between Cu(II) and catechins, particularly synthetic analogs of catechin, have been poorly studied.

Previously, we have demonstrated that 3-*O*-octanoyl-(+)-catechin had similar free radical scavenging activity, superior antioxidative activity in lipid peroxidation, but inferior antioxidative activity in protein oxidation to (+)-catechin.[7)] The difference in the affinity of catechins for target molecules is supposed to contribute to the diversity in antioxidative capacity. In the present study, we first compared the antioxidative capacity of 3-*O*-acyl derivatives of (+)-catechin with (+)-catechin in protein oxidation induced by a radical generator, and then examined the interaction between catechins and Cu(II) ions in oxidative protein damage. The formation of carbonyl groups in bovine serum albumin (BSA) was used as a marker of oxidative protein damage because protein carbonyls are generated by a number of alkoxyl and peroxyl radical reactions.[8)]

Recently, intense public interest in the safety of so-called health foods is growing with their increasing demand. Catechins and copper, which are assumed to act as antioxidative factors in living cells, are used in Japan as ingredients of dietary supplements and foods with nutrient function claims, respectively. The aim of this study was to obtain information about chemical interactions of dietary constituents *in vitro*.

MATERIALS AND METHODS

Reagents —— 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), BSA, (+)-catechin, $CuCl_2 \cdot 2H_2O$, and $(CH_3COO)_2Zn \cdot 2H_2O$ were purchased from Wako Pure Chemicals, Osaka, Japan. 3-*O*-Acyl derivatives of (+)-catechin were synthesized and purified as described in a previous report.[9] Other chemicals were of reagent grade.

Assay for the Amounts of Carbonyl Groups in BSA —— The formation of protein carbonyl groups *in vitro* was initiated by adding 0.3 ml of 100 mM AAPH solution to 2.4 ml of BSA solution (1.25 mg/ml 100 mM phosphate buffer, pH 7.4) and 0.3 ml of test compound solutions. (+)-Catechin, 3-*O*-butyryl-(+)-catechin, 3-*O*-octanoyl-(+)-catechin, and 3-*O*-palmitoyl-(+)-catechin were dissolved in 10% dimethylsulfoxide. The reaction mixture was incubated at 37°C for 90 min, then the amounts of protein carbonyl groups were determined by the method of Levine *et al.*[10] with a slight modification. One milliliter of the reaction mixture was removed to a glass tube, and blended with 0.5 ml of 10 mM 2,4-dinitrophenylhydrazine in 2.5 M HCl. The mixture was left for 1 hr at room temperature. Then 0.5 ml of 20% trichloroacetic acid solution was added to the tube, and the tube was left in ice bucket for 10 min, followed by centrifugation at $3000 \times g$ for 10 min. After the supernatant was discarded, the protein pellets were washed three times with 3 ml ethanol : ethylacetate (1 : 1, v/v) and dissolved in 2 ml of 6 M guanidine (pH 2.3) with vortex mixing, and then absorbance at 370 nm was measured. The carbonyl content was calculated using a molar absorption coefficient of $22000\ M^{-1}\ cm^{-1}$. Antioxidative activity was evaluated on the basis of the number of carbonyl groups formed expressed as a percentage of control.

Assay for ABTS Radical Cation Scavenging Activity —— Free radical scavenging activity of (+)-catechin and 3-*O*-acyl-(+)-catechins was determined by the method in a previous report.[7] The absorbance of ABTS radical cations at 734 nm was measured 7 min after mixing 2 ml of the solution of ABTS radical cations with 20 μl of a test compound solution in a cuvette. Catechins were dissolved in ethanol, and the catechin solution was replaced with vehicle in a control experiment. The residual ABTS radical cations in the test solution were expressed as a percentage of the control based on the absorbance.

Statistical Analysis —— All values are expressed as the mean ± S.E. Differences among groups were analyzed by Tukey-Kramer's test, and were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Proteins contain several kinds of amino acid residues liable to be attacked by oxygen free radicals, yielding carbonyl groups in proteins and fragments of peptides.[8,11] The addition of AAPH stimulated the formation of carbonyl groups in BSA time-dependently (Fig. 1A). Oxidation of BSA was significantly antagonized by the addition of 0.1 mM (+)-catechin and its 3-*O*-acyl derivatives (Fig. 1B). The number of carbon atoms of acyl chain did not make a significant difference in antioxidant activity of 3-*O*-acyl derivatives of (+)-catechin. Neither of the catechins tested at 0.01 mM had significant effects on the formation of carbonyl groups induced by AAPH (data not shown). These catechins showed less antioxidative potential against proteins than lipids in our previous report. In the present experiments, 1.0 mg/ml BSA, corresponding to about 0.015 mM BSA, was used. So, masking the radical scavenging activity of catechin by BSA[12] may partly be concerned with the weak antioxidant activity of these catechins.

Several studies have reported that polyphenols chelate metal ions, enhanced the antioxidative ef-

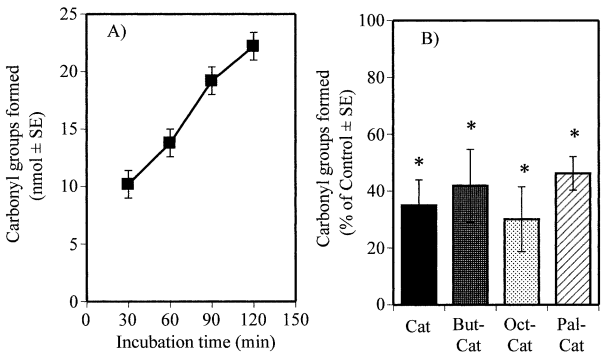

Fig. 1. Formation of Carbonyl Groups in BSA by AAPH, and Effects of (+)-Catechin and Its 3-*O*-Acyl Derivatives on Protein Carbonyl Formation

A) BSA solution was incubated in the presence of 10 mM AAPH for 30 to 120 min at 37°C. B) BSA solution was incubated in the presence of AAPH together with 0.1 mM (+)-catechin (Cat), 0.1 mM 3-*O*-butyryl-(+)-catechin (But-Cat), 0.1 mM 3-*O*-octanoyl-(+)-catechin (Oct-Cat), or 0.1 mM 3-*O*-palmitoyl-(+)-catechin (Pal-Cat) for 90 min at 37°C. Each bar represents the mean ± S.E. from 4 separate experiments. *, statistically different from control at $p < 0.05$ by Tukey-Kramer's test.

Table 1. Effects of Catechins and Metal Ions on the Formation of Carbonyl Groups in BSA in the Presence of AAPH

Carbonyl groups formed (% of Control ± S.E.)		Carbonyl groups formed (% of Control ± S.E.)	
Experiment I (n = 4)		Experiment III (n = 3)	
Cat	32.3 ± 1.4*	Oct-Cat	29.4 ± 0.4*
Cu^{2+}	61.6 ± 2.9*	Cu^{2+}	71.5 ± 0.9*
Cat + Cu^{2+}	104.7 ± 11.1	Oct-Cat + Cu^{2+}	146.0 ± 0.3*
Experiment II (n = 3)		Experiment IV (n = 3)	
Cat	33.0 ± 3.5*	Oct-Cat	29.4 ± 0.0*
Zn^{2+}	81.0 ± 4.0*	Zn^{2+}	78.2 ± 0.0*
Cat + Zn^{2+}	38.6 ± 4.3*	Oct-Cat + Zn^{2+}	28.0 ± 0.0*

In a control experiment, BSA (1.0 mg/ml) in 80 mM phosphate buffer (pH 7.4) in the presence of 10 mM AAPH was incubated for 90 min at 37°C. The concentrations of (+)-catechin (Cat), 3-*O*-octanoyl-(+)-catechin (Oct-Cat), $CuCl_2 \cdot 2H_2O$, and $(CH_3COO)_2Zn \cdot 2H_2O$ were 0.1 mM each. Experiments I to IV were separately carried out. Data are expressed as % of control ± S.E. from 3–4 experiments. *, statistically different from control at $p < 0.05$ by Tukey-Kramer's test.

fects of polyphenols.[13, 14] In the present study, however, 0.1 mM Cu(II) ions, but not 0.1 mM Zn(II) ions, distinctly blocked antioxidative effects of 0.1 mM (+)-catechin and 3-*O*-octanoyl-(+)-catechin on BSA oxidation induced by AAPH, though these metal ions by themselves significantly inhibited the formation of carbonyl groups (Table 1). It seems possible that not only ionic interactions but also the reduction of copper, a transition metal, are involved differently from zinc, a typical element. Since the hydroxyl groups of catechins are plausibly responsible for both radical scavenging activity and binding sites for metal ions,[3] we examined the effects of Cu(II) ions on ABTS radical cation scavenging activity of catechins. The coexistence of equimolar Cu(II) ions with 0.1 mM catechins had no effects on radical scavenging activities of (+)-catechin and 3-*O*-octanoyl-(+)-catechin (Fig. 2). Accordingly, reducing the antioxidant effects of catechins by Cu(II) ions on BSA oxidation is not explained in terms of the alteration of radical scavenging activities.

Then we incubated BSA solution (1.0 mg/ml) with catechins and Cu(II) ions in the absence of AAPH. The coexistence of (+)-catechin or 3-*O*-octanoyl-(+)-catechin with Cu(II) ions drastically increased the formation of carbonyl groups (Fig. 3). Replacement of C3-hydroxyl group of (+)-catechin with C3-*O*-octanoyl group did not show much difference in protein oxidation from (+)-catechin. When the concentration of (+)-catechin was fixed at 0.1 mM, the formation of carbonyl groups was augmented with an increase in the concentration of Cu(II) ions (Fig. 4). (+)-Catechin also increased the number of carbonyl groups in a dose-dependent

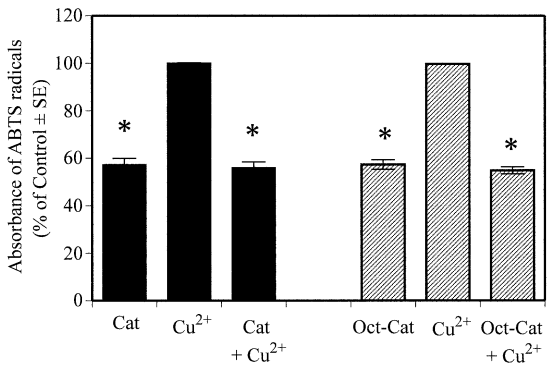

Fig. 2. Radical Scavenging Activity of Catechins

Absorbance of ABTS radicals after mixing with test compounds is indicated as % of control. In a control experiment, vehicle was added instead of 0.1 mM (+)-catechin (Cat), 0.1 mM 3-*O*-octanoyl-(+)-catechin (Oct-Cat), or 0.1 mM $CuCl_2 \cdot 2H_2O$. Each bar represents the mean ± S.E. from 4 separate experiments. *, statistically different from control at $p < 0.05$ by Tukey-Kramer's test.

manner when the concentration of Cu(II) ions was fixed at 0.1 mM (Fig. 4).

BSA has been reported to make complexes with Cu(II) ions and Zn(II) ions.[15] The sequestration of Cu(II) ions by macromolecules in living cells is likely to contribute to the inactivation of hazardous effects of metal ions.[16] It is currently unclear whether Cu(II) ions in bound form are inactive in protein oxidation. Cao *et al.*[5] have indicated using oxygen radical absorbance capacity assay that flavonoids act as prooxidant in the presence of Cu(II) ions. Akagawa and Suyama[17] have reported that polyphenols in the presence of Cu(II) ions converted the lysine residue of BSA to α-amino-adipic semialdehyde residue by oxidative deamination. Additionally, Hayakawa *et al.*[18] have

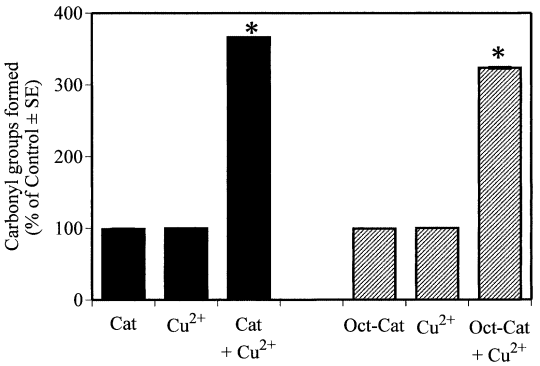

Fig. 3. Protein Carbonyl Formation in the Absence of AAPH

BSA solution was incubated with test compounds for 90 min at 37°C. (+)-Catechin (Cat), 3-*O*-octanoyl-(+)-catechin (Oct-Cat), or $CuCl_2 \cdot 2H_2O$ was used at 0.1 mM. In a control experiment, BSA solution was incubated with vehicle instead of test compounds. Formation of carbonyl groups is indicated as % of control. Each bar represents the mean ± S.E. from 3 separate experiments. *, statistically different from control at $p < 0.05$ by Tukey-Kramer's test.

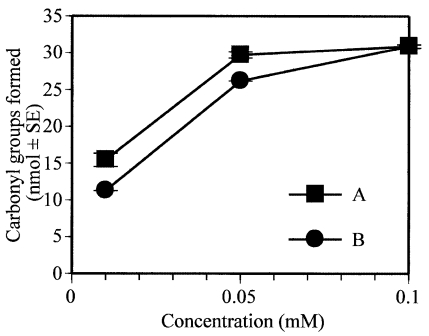

Fig. 4. Dose-dependent Formation of Carbonyl Groups in the Absence of AAPH

A: BSA solution in the presence of 0.1 mM (+)-catechin was incubated with 0.01, 0.05, and 0.1 mM $CuCl_2 \cdot 2H_2O$ for 90 min at 37°C. Each point represents the mean ± S.E. from 3 separate experiments; B: BSA solution in the presence of 0.1 mM $CuCl_2 \cdot 2H_2O$ was incubated with 0.01, 0.05, and 0.1 mM (+)-catechin for 90 min at 37°C. Each point represents the mean ± S.E. from 3 separate experiments.

demonstrated that the incubation of catechins with Cu(II) ions under aerobic conditions generated hydrogen peroxide and hydroxyl radical. These findings are suggestive of the formation of carbonyl groups in BSA in the presence of catechins together with Cu(II) ions in our study. In addition, BSA probably weakened the radical scavenging activity of catechins by binding them.[12] Hence, Cu(II) ions could convert (+)-catechins from an antioxidant to a prooxidant in protein oxidation. However, further work is needed to clarify mechanisms including the participation of reactive oxygen species and to elucidate the biological or pharmacological effects induced by protein oxidation *in vivo*.

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