(+)-Catechin with Cu^{2+} Induces Protein Modifications via Reactive Oxygen Species-independent Pathway

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Catechins show various biological activities, for which beneficial as well as adverse effects have been observed. Unfavorable properties of catechins are often attributed to reactive oxygen species (ROS) production. Our aim was to investigate whether catechins affect protein via ROS generation. We examined the effects on bovine serum albumin (BSA) of (+)-catechin with Cu^{2+} as well as ascorbate with Cu^{2+}. The ascorbate/Cu^{2+} system is a metal-catalyzed oxidation (MCO) system and can produce ROS. Both systems altered spectroscopic properties of albumin, increased protein carbonyl content and resulted in several conformational changes. Catalase and hydroxyl radical scavengers markedly suppressed protein modifications by the ascorbate/Cu^{2+} system but not those by the (+)-catechin/Cu^{2+} system. As far as we observed, no protein alteration induced by the (+)-catechin/Cu^{2+} system correlated with ROS formation.

Key words —— (+)-catechin, copper, protein modification, reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$), are generated continuously during physiological processes. Uncontrolled ROS are detrimental to biomolecules.[1] Accumulated evidence, on the other hand, reveals that ROS play essential roles in cellular redox events. Some ligand-receptor interactions produce ROS, which subsequently initiate specific signal transduction,[2–4] attracting more attention. In such attempts, the nonenzymic metal-catalyzed oxidation (MCO) system, composed of oxygen, trace metals (Fe^{3+} or Cu^{2+}) and an electron donor (*e.g.*, ascorbate and glutathione), has been widely used as a model ROS formation system. It is assumed that this system attacks metal-binding sites on proteins, yielding site-specific modifications.[5]

Catechins are plant phenolic compounds with a variety of activities.[6] Whilst the concept that catechins are potent antioxidants is now broadly accepted, catechins show cytotoxic and pro-oxidant effects after ROS production under certain conditions, particularly in the presence of Cu^{2+}.[7–11] For this reason, the unfavorable properties of catechins often correlate with their ROS generative capacity. The available data indicate that catechins with gallyl group have stronger ROS production abilities.[12, 13] Nevertheless, we previously observed that (+)-catechin, lacking that moiety, accelerated protein carbonylation in the presence of Cu^{2+}.[14]

In the present study, we focused on the (+)-catechin/Cu^{2+} system. The effects of this system on bovine serum albumin (BSA) were characterized and then compared to those by the ascorbate/Cu^{2+} system, extensively utilized as the MCO system. It was discussed whether the protein modifications induced by (+)-catechin with Cu^{2+} depend on ROS formation.

MATERIALS AND METHODS

Materials —— Fatty acid-free BSA, (+)-catechin, L-(+)-ascorbic acid sodium salt, copper(II) chloride dihydrate, catalase from bovine liver (11000 units/ml), superoxide dismutase from bovine erythrocyte (SOD, 30000 units/ml) and all other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification. All solutions were dissolved in water with the following exceptions: BSA, catalase, SOD and trypsin in 100 mM phosphate buffer (pH 7.4), (+)-catechin in 10% dimethylsulfoxide, and *p*-nitrophenyl acetate (NPA) in 5% acetonitrile.

Preparations of Treated BSA —— BSA was incubated at 37°C in the (+)-catechin/Cu^{2+} or ascorbate/Cu^{2+} system. The final concentrations of BSA and other compounds were 1 mg/ml (*ca.*

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15 μM) and 0.1 mM, respectively.

Spectroscopic Studies of Treated BSA —— Test solutions were passed through a prepacked PD-10 column (Sephadex G-25, GE Healthcare UK Ltd., Buckinghamshire, U.K.) and eluted with phosphate buffer (pH 7.4). The fraction containing the proteins was collected and its absorption spectrum was recorded at 37°C. Fluorescence was monitored at 275 nm excitation. Protein concentration of each sample was measured by Bradford's method and standardized.

Determination of Protein Carbonyl Content —— Protein carbonyls were quantified by converting carbonyl groups into hydrazone conjugates with 2,4-dinitrophenyl hydrazine. The procedures followed our previous report.[14)]

Evaluation of Conformational Changes in Treated BSA —— Protein solubility and susceptibility to proteolysis were estimated as described.[15, 16)] In the former case, the sample solution (0.5 ml), prepared in the same way as for spectroscopic studies, was diluted with 3.0 M KCl-50 mM succinate buffer (2.0 ml, pH 4.0) and left on ice for 60 min. Next, the test tube was centrifuged at 2000 rpm for 10 min and the remaining soluble protein was determined by Bradford's assay. In the latter case, the sample (3.0 ml) was incubated at 37°C with trypsin solution (56 μg/ml, 0.3 ml) for 90 min. The incubation mixture (1.0 ml) and 10% trichloroacetic acid (TCA, 1.0 ml) was then mixed. After 15-min centrifugation at 3000 rpm, TCA-soluble peptides were measured by Bradford's method. In addition, we evaluated protein local environmental changes following treatment with 4,4′-bis-1-anilinonaphthalene-8-sulfonate (bis-ANS) affinity and esterase-like activity, as described.[17, 18)] Briefly, the fluorescence of bis-ANS and absorbance of *p*-nitrophenol, which resulted from the rapid reaction between BSA and NPA, were monitored.

Statistical Analysis —— Values are expressed as the mean ± S.D. All assays were repeated over three times and statistical differences were assessed using one-way analysis of variance (ANOVA).

RESULTS

Spectra of Treated BSA

UV spectra for native and 90-min-treated BSA indicated that both (+)-catechin/Cu^{2+} and ascorbate/Cu^{2+} systems had hyperchromic effects (Fig. 1A). The two systems also decreased fluorescence intensities and caused blue-shift of the emission spectra (Fig. 1B). In short, the native ordered structure and tryptophan environment were altered with each treatment. Furthermore, the addition of catalase or mannitol, a well-known HO˙ scavenger, abolished the effects of the ascorbate/Cu^{2+} system, but not the (+)-catechin/Cu^{2+} system (data not shown).

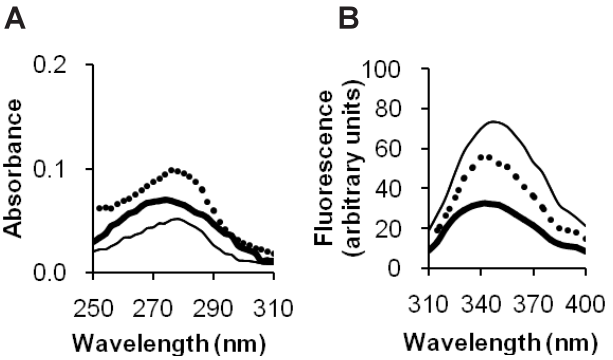

Fig. 1. Spectroscopic Modifications with Treatment

(A) Absorbance spectra and (B) fluorescence emission spectra at λ_{ex} = 275 nm of BSA, untreated (——) and incubated for 90 min with (+)-catechin/Cu^{2+} (——) or ascorbate/Cu^{2+} (·····).

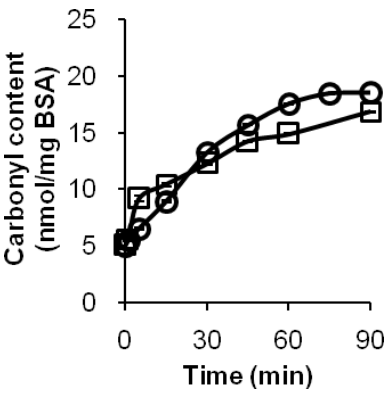

Fig. 2. Time Course of Carbonyl Content of Treated BSA

BSA was exposed to (+)-catechin/Cu^{2+} (○) or ascorbate/Cu^{2+} (□). Aliquots were withdrawn at different time intervals and protein carbonyls were measured.

Protein Carbonyl Content

In two systems, (+)-catechin/Cu^{2+} and ascorbate/Cu^{2+}, we observed time-dependent protein carbonyl formation (Fig. 2). These results agree with the concept that protein oxidation often accompanies the accumulation of protein carbonyls.[19)] To assess the potential factors in protein carbonyl production, catalase or HO˙ scavenger (mannitol or thiourea) was incubated with each system (Table 1). Catalase, mannitol and SOD gave no protection against the (+)-catechin/Cu^{2+}

system; however, thiourea partially suppressed protein carbonyl production. Thiourea has been shown to act not only as a $HO^{\cdot}$ scavenger but also as a Cu^{+} chelator.[20] Additionally, the inhibitory effect of bathocuproinedisulfonic acid (BCS), a specific chelator of Cu^{+}, was similar to that of thiourea; thus, thiourea lowered the protein carbonyl content by chelating Cu^{+}. By contrast, in the ascorbate/Cu^{2+} system, catalase, mannitol and thiourea were all efficient inhibitors.

Table 1. Effects of Catalase and $HO^{\cdot}$ Scavengers on Protein Carbonyl Formation

Additive compounds	Carbonyl content (nmol/mg BSA)	
	(+)-Catechin/Cu^{2+}	Ascorbate/Cu^{2+}
—	18.3 ± 0.3	17.8 ± 0.2
Catalase (150 units/ml)	18.2 ± 0.2	8.37 ± 0.1[b]
Heat-inactive catalase	18.3 ± 0.3	17.9 ± 0.1
Mannitol (1.0 mM)	18.3 ± 0.3	8.00 ± 0.1[b]
Thiourea (1.0 mM)	12.4 ± 0.2[a]	7.26 ± 0.2[b]
BCS (1.0 mM)	11.9 ± 0.2[a]	
SOD (150 units/ml)	18.3 ± 0.1	

BSA was incubated in the (+)-catechin/Cu^{2+} or ascorbate/Cu^{2+} system with the additive agent. After 90-min treatment, protein carbonyl content was determined. *a*) $p < 0.01$ *v.s.* exposed to (+)-catechin/Cu^{2+} alone. *b*) $p < 0.01$ *v.s.* exposed to ascorbate/Cu^{2+} alone.

Conformational Changes

To estimate the alteration in the albumin structure, we first examined the loss of salt solubility and proteolytic resistance. Previous studies have revealed that protein oxidation or denaturation could decrease solubility in high salt buffer and enhance susceptibility to tryptic digestion.[15, 16, 18, 21] As shown in Fig. 3A and 3B, the gradual decrease in salt solubility and increase in proteolytic susceptibility were induced by the (+)-catechin/Cu^{2+} or ascorbate/Cu^{2+} system. Both treatments might cause BSA small conformational changes, which was also confirmed by the fact that fluorescence intensities were diminished by 45% and 25% after 90-min incubation with (+)-catechin/Cu^{2+} and ascorbate/Cu^{2+} systems, respectively (Fig. 1B).

Second, we studied more local conformational changes following the two treatments. Bis-ANS binds to serum albumin to enhance its fluores-

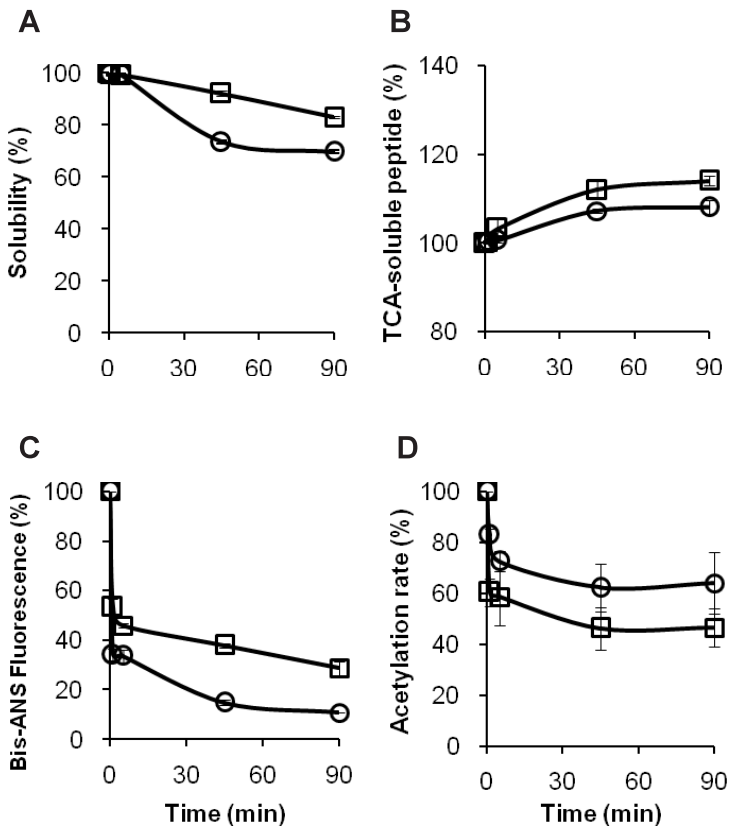

Fig. 3. Conformational Alterations with Treatment

Time courses of (A) solubility in 3.0 M KCl-succinate buffer (pH 4.0), (B) susceptibility to tryptic digestion, (C) affinity with bis-ANS and (D) esterase-like activity. BSA was exposed to (+)-catechin/Cu^{2+}(○) or ascorbate/Cu^{2+}(□). Aliquots were withdrawn at specified times and used for each study. Results are expressed as percent values of untreated BSA.

Table 2. Inhibitory Experiments for Conformational Modifications

Additive compounds	Solubility (%)		TCA-soluble peptide (%)	
	(+)-Catechin/Cu^{2+}	Ascorbate/Cu^{2+}	(+)-Catechin/Cu^{2+}	Ascorbate/Cu^{2+}
—	69.7 ± 0.5	82.9 ± 0.4	108.1 ± 1.6	114.0 ± 1.1
Catalase (150 units/ml)	70.6 ± 1.8	99.5 ± 0.3[a)]	108.3 ± 1.2	100.7 ± 0.4[a)]
Heat-inactive catalase	70.9 ± 1.6	83.2 ± 0.8	107.7 ± 0.3	115.3 ± 1.4
Mannitol (1.0 mM)	69.7 ± 0.4	99.5 ± 0.4[a)]	108.7 ± 1.0	100.1 ± 0.3[a)]
Additive compounds	Bis-ANS Fluorescence (%)		Acetylation rate (%)	
	(+)-Catechin/Cu^{2+}	Ascorbate/Cu^{2+}	(+)-Catechin/Cu^{2+}	Ascorbate/Cu^{2+}
—	10.9 ± 0.2	28.8 ± 2.8	64.0 ± 12.3	46.5 ± 7.6
Catalase (150 units/ml)	10.9 ± 0.4	96.5 ± 1.6[a)]	64.6 ± 11.5	81.6 ± 11.7[a)]
Heat-inactive catalase	10.8 ± 0.4	29.2 ± 3.3	66.5 ± 11.3	45.5 ± 7.2
Mannitol (1.0 mM)	10.8 ± 0.3	98.2 ± 1.0[a)]	64.0 ± 8.2	87.1 ± 8.8[a)]

BSA was incubated for 90 min in the (+)-catechin/Cu^{2+} or ascorbate/Cu^{2+} system with the additive compound. *a)* $p < 0.01$ *v.s.* the respective group treated with ascorbate/Cu^{2+} alone.

cence quantum yield, whose intensity, *i.e.*, the affinity for this probe, is lowered with albumin denaturation.[22,23] In either system under study, bis-ANS affinity exhibited an extremely rapid decrease, suggesting modification at the dye binding sites (Fig. 3C). From the view of enzymatic activity of BSA, there was additional evidence of partial modification of the structure. Serum albumin possesses esterase activity, which requires protein conformational integrity, especially the subdomain IIIA sequence containing tyrosine 411.[24] Significant loss of catalytic ability was induced by exposure to either the (+)-catechin/Cu^{2+} or ascorbate/Cu^{2+} system (Fig. 3D).

As shown in Table 2, the advance addition of catalase or mannitol exerted no inhibitory action upon the conformational changes caused by the (+)-catechin/Cu^{2+} system. In contrast, these inhibitors strongly hindered the ascorbate/Cu^{2+} system.

DISCUSSION

(+)-Catechin with Cu^{2+} altered the properties of BSA. This (+)-catechin/Cu^{2+} system enhanced absorbance, lowered fluorescence intensities with a blue shift and accelerated protein carbonylation. Additionally, the treated BSA exhibited a loss of salt solubility, proteolytic resistance, affinity with an apolar probe (bis-ANS) and esterase-like activity. These observations, showing changes in the conformational state and the environment surrounding two tryptophan (Trp) residues, Trp 134 and Trp 212, suggest that BSA became more unfolded and hydrophobic. Trp 212, located in the subdomain IIA pocket, plays a pivotal role in the interaction with site I-binding compounds.[24] Besides delivering a variety of ligands, serum albumin is crucial in biological processes such as maintaining oncotic pressure. Considering that these activities greatly require an intact form of albumin,[24] the observed modifications by the (+)-catechin/Cu^{2+} system could accompany protein functional changes.

In contrast to the model MCO system, the (+)-catechin/Cu^{2+} system was unaffected by catalase or $HO^{\cdot}$ scavengers. No effect induced by the (+)-catechin/Cu^{2+} system was dependent on H_2O_2 or $HO^{\cdot}$ production. The MCO system, composed of oxygen, Fe^{3+} or Cu^{2+} and an appropriate electron donor, is characterized by site-specific ROS generation.[25] Although (+)-catechin has redox potential low enough to act as an electron donor,[26–29] previous reports agree that the ROS-generating ability of (+)-catechin is markedly lower than that of other catechins, such as (−)-epigallocatechin and (−)-epigallocatechin gallate.[12,13] The inhibitory experiments indeed support that ROS production by (+)-catechin is insignificant, further implying that O_2 is not involved in electron transfer from that donor. Collectively, our results show the (+)-catechin/Cu^{2+} system as distinct from the typical MCO system.

In conclusion, the (+)-catechin/Cu^{2+} system can yield alterations to BSA by a process other than ROS formation. Some catechins have been found to cause DNA damage and lipid peroxidation under certain conditions, especially in the presence of Cu^{2+}.[7–11] Such prooxidative properties, as well as their actions on cell signal transduction, are often attributed to ROS, since most effects occurred with ROS generation, thereby being affected by catalase and $HO^{\cdot}$ scavengers.[7–11,30–34] In contrast, as in the present cases, several effects, for instance,

(−)-catechin gallate-induced apoptosis and flavonol myricetin-mediated DNA damage, have not been suppressed by the above ROS inhibitors.[35,36] To understand these structural differences, we now examine still more potential factors such as the affinity with biomolecules. Our results highlighted the ROS-independent pathway, which might be another strategy to elucidate the molecular mechanisms underlying the activities of catechins.

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