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# Structural characteristics of green tea catechins for formation of protein carbonyl in human serum albumin

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ABSTRACT

Catechins are polyphenolic antioxidants found in green tea leaves. Recent studies have reported that various polyphenolic compounds, including catechins, cause protein carbonyl formation in proteins via their pro-oxidant actions. In this study, we evaluate the formation of protein carbonyl in human serum albumin (HSA) by tea catechins and investigate the relationship between catechin chemical structure and its pro-oxidant property. To assess the formation of protein carbonyl in HSA, HSA was incubated with four individual catechins under physiological conditions to generate biotin-LC-hydrazide labeled protein carbonyls. Comparison of catechins using Western blotting revealed that the formation of protein carbonyl in HSA was higher for pyrogallol-type catechins than the corresponding catechol-type catechins. In addition, the formation of protein carbonyl was also found to be higher for the catechins having a galloyl group than the corresponding catechins lacking a galloyl group. The importance of the pyrogallol structural motif in the B-ring and the galloyl group was confirmed using methylated catechins and phenolic acids. These results indicate that the most important structural element contributing to the formation of protein carbonyl in HSA by tea catechins is the pyrogallol structural motif in the B-ring, followed by the galloyl group. The oxidation stability and binding affinity of tea catechins with proteins are responsible for the formation of protein carbonyl, and consequently the difference in these properties of each catechin may contribute to the magnitude of their biological activities.



## 1. Introduction

Epidemiological studies have revealed that the consumption of green tea may protect against several risk factors for diseases such as cancer, diabetes, and vascular disease.[1–3] Its diverse biological activities have been attributed to a group of polyphenol compounds, namely catechins.[4] The major catechins of green tea extract are (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg), as shown in Figure 1. The number of hydroxyl groups on the B-ring and the presence or absence of a galloyl group are responsible for differences in the structure and function of each catechin. These catechins have been found to act as free radical scavengers and have been widely studied for their antioxidant activity in vitro.[5] In contrast, a wealth of data suggests that most of the relevant mechanisms of disease prevention attributed to catechins, such as in cancer, are not related to their antioxidant properties, but rather are due to their pro-oxidant action and the direct interaction of catechins with target proteins.[6–8]

Several lines of evidence indicate that *O*-diphenolic compounds such as tea catechins have poor stability in neutral or alkaline solutions. Previous reports showed that catechol- and pyrogallol-type polyphenols were easily oxidized in a cell culture medium with slightly alkaline pH.[9,10] Oxidation of catechins with an *O*-diphenolic structural motif in their B-ring leads to the formation of a corresponding quinone and the gain of pro-oxidant activities. For example, in slightly alkaline solutions, catechol- and pyrogallol-type catechins undergo autoxidation to form reactive oxygen species (ROS), including superoxide ($O_2^-$) and hydrogen peroxide, resulting in polymerization and decomposition.[11,12] In addition, electrophilic flavonoid quinone rapidly react with sulfhydryls in reduced glutathione or protein cysteine residues to form cysteinyl adducts.[13,14] More recently, we found that 3,4-dihydroxyphenyl acetic acid and EGCg form covalent adducts with protein sulfhydryls through autoxidation.[15] ROS and electrophiles can cause oxidative modifications to sensitive proteins that can lead to changes in protein function.[16,17]

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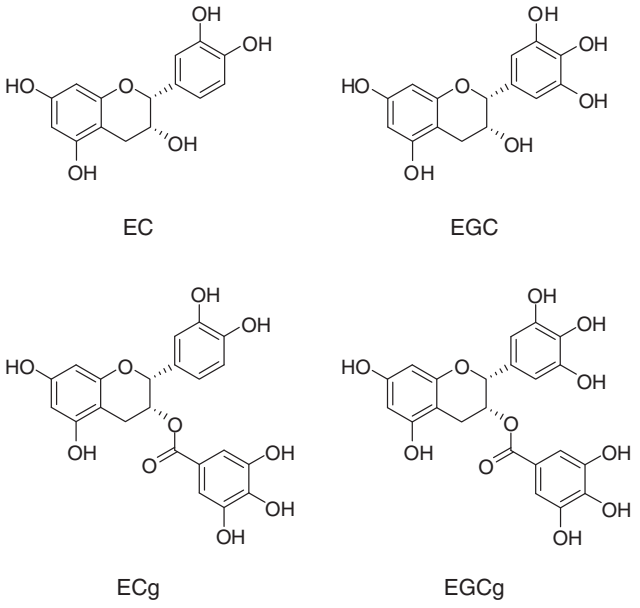


**Figure 1.** Chemical structures of tea catechins.

Protein oxidation has emerged as a potential mechanism of dynamic, post-translational regulation of a variety of regulatory, structural, and metabolic proteins.[18,19]

Deamination is an oxidative reaction that occurs under aerobic conditions in all tissues. During oxidative deamination, an amino acid is converted into the corresponding keto acid by the removal of the amine functional group as ammonia and the amine functional group is replaced by the ketone group. Amine oxidase catalyzes the oxidative deamination of $\varepsilon$-amino groups of lysine residues to form $\alpha$-aminoadipic $\delta$-semialdehyde (AAS) residues, which are precursors of cross-links and are required for proper cross-linking of elastin and collagen.[20] AAS is one of the most general protein carbonyls and is broadly observed among plasma, normal tissue, and abnormal tissue.[21,22] Although protein carbonyl was generally known as a marker of oxidative stress, a recent study showed the physiological importance of the formation in vivo.[23] Previously, administration of catechins has been reported to elevate amine oxidase activity in chick aorta, increase collagen stability, and protect against lathyrism induced by a specific inhibitor of the enzyme.[24] Moreover, it has been reported that the incubation of polyphenol-rich beverages, such as green tea, with bovine serum albumin (BSA) at a physiological pH and temperature converts the lysine residues of BSA to AAS and the formation of protein carbonyl was caused by the catechins contained within green tea, black tea, and coffee.[25] These findings revealed that polyphenol has amine oxidase-like activity, and provides a mechanism for oxidative deamination by polyphenols involving quinone-mediated oxidation. Thus, oxidation of catechins and the resulting protein carbonylation is thought to be responsible for certain biological activities. However, the relationship between the chemical structure of catechins and its amine oxidase-like activity with oxidative deamination has not yet been characterized in detail.

Human serum albumin (HSA), the most abundant protein in blood, is a 66-kDa protein that plays an important role in the reversible binding of many compounds. Several recent studies have suggested that catechins form complexes with albumin for transport in human blood, and in this study, their binding affinity to albumin is believed to modulate their bioavailability. We have previously shown that HSA stabilizes EGCg by the formation of a complex, involving non-covalent interactions, in human serum and a neutral buffer solution, and that protein carbonyl is formed in HSA during the prevention of EGCg oxidation.[26] HSA is an attractive target for protein carbonylation because it is highly abundant, constituting over half the total serum protein. Therefore, HSA may be one of the targets of protein carbonylation by catechins.

In this study, we evaluate the formation of protein carbonyl in HSA by tea catechins and their analogs, and investigate the relationship between the chemical structure of catechin and its prooxidant property.

## 2. Materials and methods

### 2.1. Chemicals

EC, EGC, ECg, and EGCg were kindly provided by Mitsui Norin Co. Ltd (Shizuoka, Japan). (−)-4′-*O*-Methyl-epigallocatechin-3-gallate (EGCg4′OMe) and (−)-epigallocatechin-3-(3″-*O*-methyl) gallate (EGCg3″OMe) were synthesized according to the method previously reported.[27] (−)-Epigallocatechin-3-(4″-*O*-methyl) gallate (EGCg4″OMe) was obtained from Nagara Science Co. Ltd (Gifu, Japan). HSA and pyrogallol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Horseradish peroxidase-conjugated streptavidin (HRP-avidin) and ECL Western blotting detection reagents were obtained from GE Healthcare UK Ltd (Buckinghamshire, UK). Biotin-LC-hydrazide was purchased from Pierce (Rockford, IL, USA). All other reagents were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan).

### 2.2. Stability of tea catechins in phosphate buffer

EC, EGC, ECg, EGCg, or three methylated catechins, at a concentration of 100 μM, were each incubated in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min. The amount of each catechin was determined by HPLC analysis as previously described.[28] Briefly, separation of the catechins was carried out on a nanospace Gulliver system (Jasco, Tokyo, Japan) with a UV detector, using a Capcell Pak C18 UG120 column (Shiseido Co. Ltd, Tokyo, Japan) with a mobile phase consisting of 20% methanol (EC and EGC) or 25% methanol (ECg, EGCg, and methylated catechins) in water with 0.1% trifluoroacetic acid. The flow rate was 1.0 mL/min, and the elution profiles were monitored by absorbance at 285 nm.

### 2.3. Biotin labeling of protein carbonyls in HSA

Biotin labeling of protein carbonyls in HSA was performed as previously reported.[29] Briefly, HSA (30.0 mg/mL) was incubated with catechins or structurally related polyphenols at a concentration of 100 μM in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min. After dilution to 1.0 mg/mL, the samples were treated with 1.0 mM biotin-LC-hydrazide at room temperature for 60 min in the dark. The samples (1.0 μg) were separated by SDS–PAGE using a 10% gel. For Western blotting, a gel was transblotted onto a PVDF membrane. The membranes were incubated with EzBlock (ATTO, Tokyo, Japan) for blocking, washed, and incubated with HRP-avidin. This procedure was followed by the addition of ECL reagents. The bands were visualized using a lumino-image analyzer (FUJIFILM Co., Tokyo, Japan).

## 3. Results

### 3.1. Formation of protein carbonyl in HSA by tea catechins

Oxidative deamination, by various polyphenolic compounds, results in the protein carbonyl formation, presumably through oxidation of polyphenols to the corresponding quinines (Fig. 2).[25] In this reaction, oxidation stability of polyphenols is important. Hence, we first examined the oxidation stability of the catechins in neutral

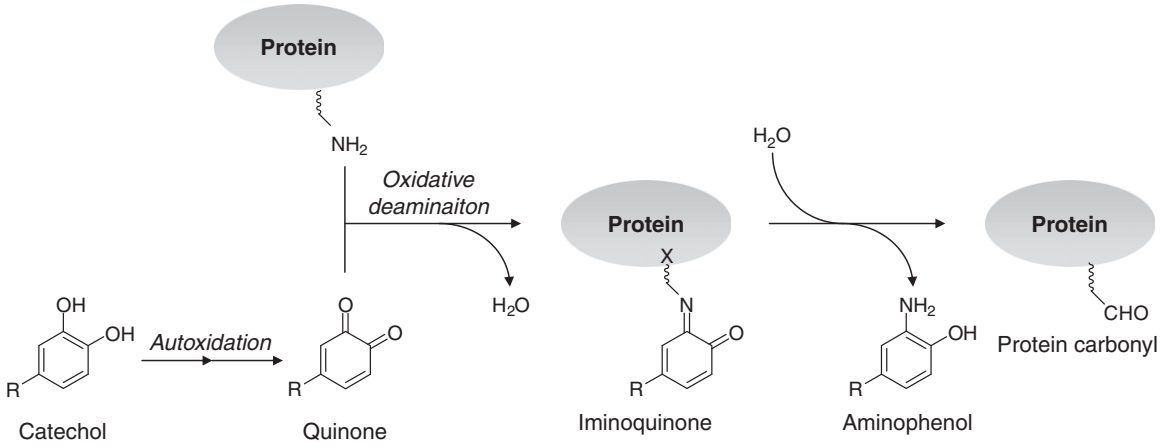


**Figure 2.** Assumed mechanism involved in oxidative deamination by catechol-type polyphenols through autoxidation.

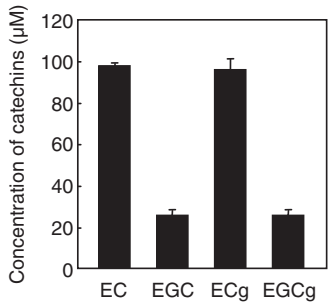


**Figure 3.** Stability of tea catechins in neutral buffer. The catechins (100 μM) were incubated in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min. The amount of each catechin was determined by HPLC analysis as described in Section 2.2.

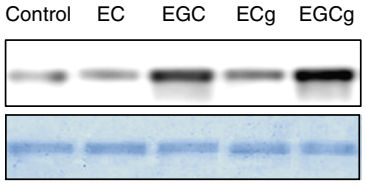


**Figure 4.** Formation of protein carbonyl in HSA by tea catechins. HSA (30.0 mg/mL) was incubated with EC, EGC, ECg or EGCg at a concentration of 100 μM in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min. The reaction mixtures were incubated with biotin-LC-hydrazide and resolved by SDS–PAGE. Biotin-labeled proteins and total protein were detected by Western blotting with HRP-avidin (*upper*) and CBB staining (*lower*), respectively.

buffer solution. While incubation of 100 μM each of the four catechins (EC, EGC, ECg, and EGCg) in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min resulted in a decrease in the concentration of pyrogallol-type catechins (EGC and EGCg), catechol-type catechins (EC and ECg) were stable for over 60 min (Fig. 3). This indicates that the stability of catechol-type catechins was higher than that of the corresponding pyrogallol-type catechins.

To assess the formation of protein carbonyl in HSA, HSA (30.0 mg/mL) was incubated with 100 μM each of the four catechins (EC, ECg, EGC, and EGCg) in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min to generate biotin-LC-hydrazide labeled protein carbonyls. Commercial HSA is generally purified from human blood. It has been reported that protein carbonyl groups in HSA were detected in human plasma from healthy subjects.[30] Therefore, the protein carbonyl can be slightly detected in the untreated HSA. From the SDS–PAGE/Western blotting using HRP-avidin, the formation of protein

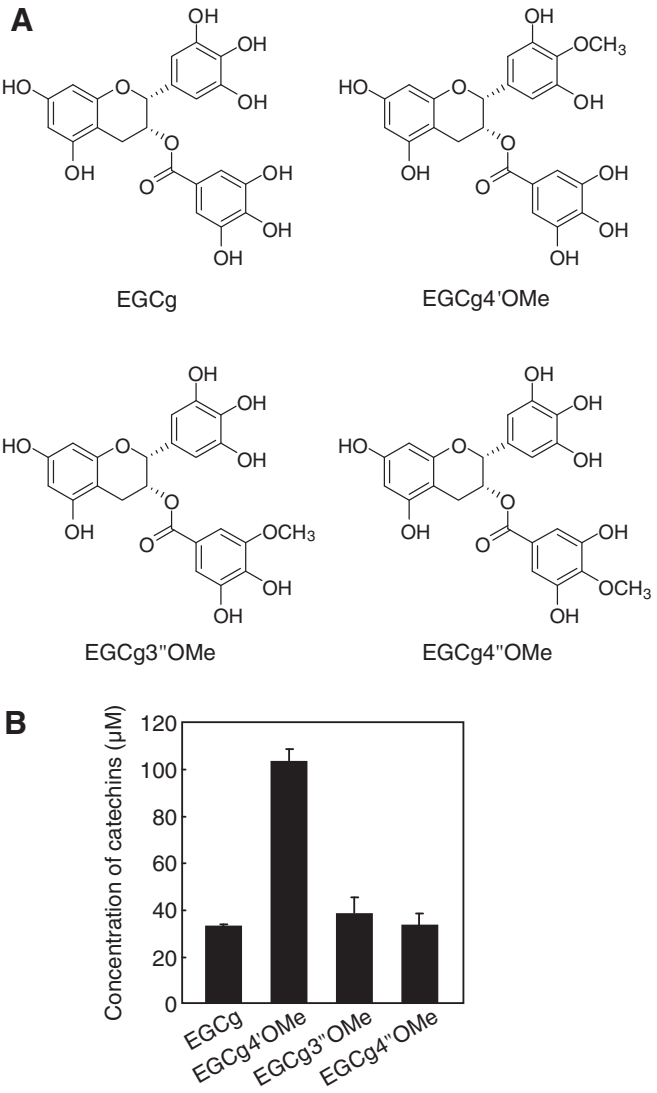


**Figure 5.** Stability of methylated catechins in neutral buffer. (A) Chemical structures of methylated catechins. (B) Comparison of the stability of methylated catechins with EGCg. The catechins (100 μM) were incubated in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min. The amount of each catechin was determined by HPLC analysis as described in Section 2.2.

carbonyl in biotin-labeled HSA was found to be higher for pyrogallol-type catechins (EGC and EGCg) than the corresponding

catechol-type catechins (EC and ECg) (Fig. 4). The protein carbonyl formation was higher for the catechins having a galloyl group (ECg and EGCg) than the corresponding catechins lacking a galloyl group (EC and EGC). These results suggest that presence of both the B-ring with the pyrogallol structure and the galloyl group affects the formation of protein carbonyl in HSA.

### 3.2. Importance of the pyrogallol structural motif in the B-ring and the galloyl group for formation of protein carbonyl in HSA by catechins

To examine the influence of the number of hydroxyl groups in catechins on the formation of protein carbonyl in HSA, the formation of protein carbonyl by methylated EGCg was compared with EGCg itself. Figure 5A shows the structures of the three methylated EGCg derivatives, EGCg4′OMe, EGCg3″OMe, and EGCg4″OMe. While incubation of 100 μM each of the three methylated catechins in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min resulted in a decrease in the concentration of EGCg3″OMe, EGCg4″OMe and EGCg, EGCg4′OMe was stable for over 60 min (Fig. 5B). This indicates that the B-ring with the pyrogallol structure, and not the galloyl group, is responsible for EGCg oxidation under neutral pH conditions.

To assess the formation of protein carbonyl by the methylated catechins, HSA (30.0 mg/mL) was incubated with 100 μM each of the four catechins (EGCg, EGCg4′OMe, EGCg3″OMe, and EGCg4″OMe) in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min to generate biotin-LC-hydrazide labeled protein carbonyls. From the SDS–PAGE/Western blotting using HRP-avidin, the formation of protein carbonyl in biotin-labeled HSA was found to be higher for EGCg than the corresponding methylated catechins (Fig. 6). The order of relative intensity of the bands is as follows: EGCg > EGCg3″OMe > EGCg4″OMe ≫ EGCg4′OMe. This result shows that the B-ring with the pyrogallol structure in EGCg, but not the galloyl group, is responsible for the protein carbonyl formation under this experimental condition. To support this result, we compared the formation of protein carbonyl in HSA by structurally related phenolic acids. Figure 7A shows the structures of 2,3-dihydroxybenzoic acid (DHBA), 3,4-DHBA, 3,5-DHBA, methylgallate, and pyrogallol. Pyrogallol and methylgallate are model compounds of the B-ring, containing a pyrogallol structure, and the galloyl group in EGCg, respectively. Formation of protein carbonyl in HSA was detected only in the incubation with pyrogallol (Fig. 7B). These results suggest that presence of the pyrogallol structural motif in the B-ring in catechins strongly influences the formation of protein carbonyl in HSA under neutral conditions.

## 4. Discussion

Much attention has recently focused on the pro-oxidant action of polyphenols because *O*-diphenolic compounds, such as tea catechins, have both poor stability and the accompanying generation of ROS, and high reactivity with protein in neutral or alkaline solutions. The formation of protein carbonyl in the presence of catechins might arise from a reaction of protein oxidative deamination attributed to the oxidation of catechins. However, the relationship between the chemical structure of catechins and its pro-oxidant action has not yet been characterized in detail. This study revealed that the important structural element contributing to the formation of protein carbonyl in HSA by tea catechins is the pyrogallol structural motif in the B-ring, followed by the galloyl group.

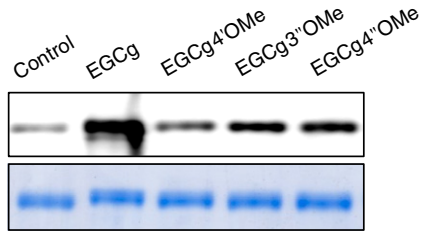


**Figure 6.** Formation of protein carbonyl in HSA by methylated catechins. HSA (30.0 mg/mL) was incubated with EGCg, EGCg4′OM, EGCg3″OMe, and EGCg4″OMe at a concentration of 100 μM in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min. The reaction mixtures were incubated with biotin-LC-hydrazide and resolved by SDS–PAGE. Biotin-labeled proteins and total protein were detected by Western blotting with HRP-avidin (*upper*) and CBB staining (*lower*), respectively.

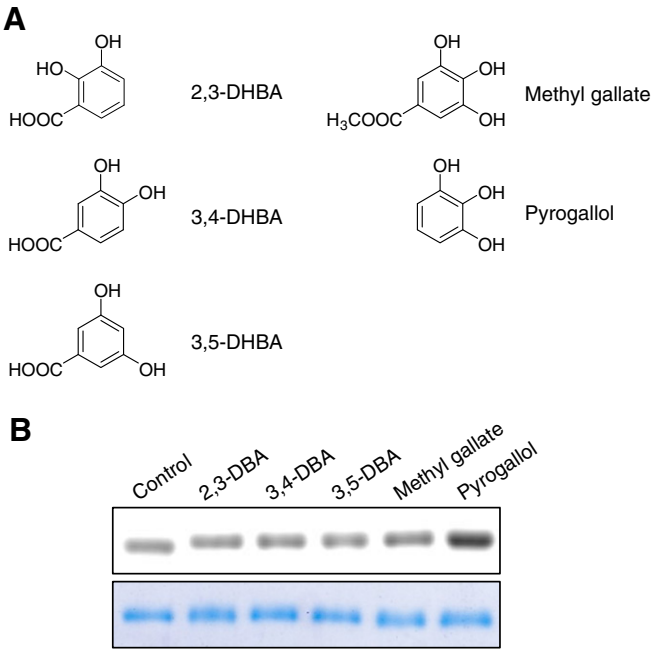


**Figure 7.** Formation of protein carbonyl in HSA by phenolic acids. (A) Chemical structures of the phenolic acids. (B) Detection of protein carbonyl in phenolic acids-treated HSA. HSA (30.0 mg/mL) was incubated with 2,3-DHBA, 3,4-DHBA, 3,5-DHBA, methylgallate, and pyrogallol at a concentration of 100 μM in 100 mM phosphate buffer (pH 7.4) at 37 °C for 60 min. The reaction mixtures were incubated with biotin-LC-hydrazide and resolved by SDS–PAGE. Biotin-labeled proteins and total protein were detected by Western blotting with HRP-avidin (*upper*) and CBB staining (*lower*), respectively.

The pyrogallol structure on the B-ring in the catechins strongly participates in the formation of protein carbonyl in HSA. In general, polyphenols having an *O*-diphenolic structure have poor stability in solutions under aerobic conditions.[9,10] Comparing four catechins (EC, EGC, ECg, and EGCg), we showed that the catechins with a pyrogallol structure on the B-ring (EGC and EGCg) are more unstable than catechins with a catechol structure on the B-ring (EC and ECg) in physiological buffer solutions (Fig. 3). In addition, we indicated that EGCg4′OMe, lacking a catechol structure on the B-ring, is stable under the same condition, although EGCg3″OMe and EGCg4″OMe, with a catechol structure on the B-ring, are unstable, as is EGCg (Fig. 5). This indicates that the B-ring with the pyrogallol structure, but not the galloyl group, is responsible for EGCg oxidation under neutral pH conditions. On the other hand, it was observed that the protein carbonyl formation was higher for the pyrogallol-type catechins (EGC and EGCg) than the corresponding catechol-type catechins (EC and ECg), as shown in Figure 4. In addition, the formation of protein carbonyl in HSA by EGCg was remarkably decreased by its *O*-methylation of the C4 hydroxyl group on the B-ring (Fig. 6). Moreover, it was observed that the protein carbonyl formation was higher for pyrogallol (model of the B-ring) than methyl gallate (model of the galloyl group), as shown in Figure 7. Previous report indicated that the stability of pyrogallol is higher than that of methyl gallate, suggesting that

the pro-oxidant action of pyrogallol is much higher than methyl gallate.[10] These results and observations strongly suggest that the pyrogallol structural motif in the B-ring is more readily oxidized than galloyl groups, and only the B-ring preferentially forms a B-ring quinone during autoxidation and consequently forms the protein carbonyl in HSA during oxidative deamination.

The galloyl group in the catechins also participates in the formation of protein carbonyl in HSA. Comparing the four catechins, we showed that protein carbonyl formation was higher for the catechins having a galloyl group (ECg and EGCg) than the corresponding catechins lacking a galloyl group (EC and EGC), as shown in Figure 4. In addition, the formation of protein carbonyl in HSA by EGCg was attenuated by *O*-methylation of the C3 or C4 hydroxyl group on the galloyl group (Fig. 6). We have previously investigated the relationship between the chemical structure of each catechin and its binding property.[31] In this study, we showed that the catechins having a galloyl group (ECg and EGCg) have higher binding affinities with HSA than the catechins lacking a galloyl group (EC and EGC), respectively. In addition, in comparison of both EGCg and methylated catechins (EGCg3″OMe and EGCg4″OMe), the number of hydroxyl groups on the galloyl group plays an important role in the interaction of catechins with HSA, and the binding affinity of tea catechins for HSA is reduced by methylation. These results and observations suggest that the interaction of catechins with HSA is important for the formation of protein carbonyl.

## 5. Conclusions

Previous structure–activity analyses of tea catechins have identified two different substructures that primarily contribute to the biological activities of the catechins. EGCg, containing both a B-ring with a pyrogallol structure and a galloyl group, exhibited higher activities than the pyrogallol structure-containing EGC or the gallate group-containing ECg in several reports, suggesting that the B-ring with a pyrogallol structure and the galloyl group in the catechins are important for induction of the biological activities.[32–34] On the other hand, protein oxidation, such as thiol oxidation and protein carbonylation, has been reported as a potential mechanism of protein function.[18,19,35] Here, we indicate that the most important structural element contributing to the formation of protein carbonyl in HSA by tea catechins is the pyrogallol structural motif in the B-ring, followed by the galloyl group. It is thought that the B-ring participates in the formation of protein carbonyl in HSA, and that the galloyl group participates in the interaction with HSA. Based on the present findings and observations, we conclude that catechins might contribute to the regulation of protein functions by pro-oxidants during oxidative deamination through autoxidation. Searching for target proteins that are carbonylated by the catechins may be the first step in understanding the molecular and biochemical mechanisms of the functional effects of catechins. We are currently extending our studies to include this line of research in our laboratory. Our findings provide the basis for understanding protein carbonylation by polyphenolic compounds.

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