Mechanisms and Structural Specificity of Hydrogen Peroxide Formation during Oxidation of Catechins

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Hydrogen peroxide (H_2O_2) formed in aqueous solutions of tea catechins such as epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg) was measured by an HPLC method. The amount of H_2O_2 formed depended on their chemical structures, pH, temperature and incubation time. The gallyl moiety in the B-ring of the gallo catechins, namely EGC and EGCg, mainly contributed to the H_2O_2 formation. Superoxide dismutase inhibited oxidation of the gallo catechins and, consequently, the H_2O_2 formation. Cytotoxic effects were investigated by a colony formation assay with Chinese hamster V79 cells, and the effects of the gallo catechins were stronger than those of EC and ECg. The cytotoxic effects of the gallo catechins were inhibited completely by catalase and partially by superoxide dismutase. These results indicate that the gallo catechins were oxidized by superoxide, accompanied by the formation of H_2O_2 , and their cytotoxic effects were ascribed to the H_2O_2 formation.

Keywords: superoxide anion, hydrogen peroxide, catechin, superoxide dismutase (SOD), epigallocatechin (EGC), epigallocatechin gallate (EGCg)

Epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg) are the major components of polyphenols in green tea infusions and are referred to as tea catechins. Activities of these catechins in growth inhibition of human lung cancer cell line, inactivation effects on human type-A influenza virus, inhibitory effects on the oxidative modification of low density lipoprotein, antibacterial activity against *Clostridium botulinum*, and lipoprotein-bound antioxidant activity, have been compared (for review see Hashimoto *et al.*, 1999). These studies indicated that the activities of ECg and EGCg were higher than those of EC and EGC. We investigated the interaction of tea catechins with lipid bilayers with liposome systems and found that the order of affinity of the catechins was as follows: ECg>EGCg>EC>EGC (Hashimoto *et al.*, 1999; Kajiya *et al.* 2001b). This means that the affinity of the gallic acid esters, namely ECg and EGCg, is higher than EC and EGC. Thus, the order of biological activities mentioned above partly reflected the order of the affinity. In other cases, however, the biological activity of EGC was equal to or higher than the other catechins in spite of having the lowest affinity among the four catechins (Saeiki *et al.* 2000). This implies that factors other than the affinity for lipid bilayers are attributable to the biological activities of EGC. EGC possesses a benzene ring with three hydroxyl groups referred to as a gallyl moiety, which has been ascribed to its antioxidant activity. In addition, EGC, EGCg and compounds with a gallyl moiety have been reported to produce hydrogen peroxide (H_2O_2) during oxidation in aqueous media (Hoshino *et al.*, 1999; Long *et al.*, 2000; Yang *et al.*, 2000). This suggests that H_2O_2 generation might be responsible for the biological activities.

The formation of H_2O_2 from (+)-catechin in aqueous solutions depended on pH, incubation time, and temperature (Nakayama *et al.*, 1995). Superoxide dismutase (SOD) inhibited both oxidation of (+)-catechin and concomitant formation of H_2O_2 . It was concluded that oxidation of (+)-catechin and the H_2O_2 formation proceeded by a chain reaction with superoxide as the chain carrier.

In this study, we investigated the effects of SOD on H_2O_2 formation during the oxidation of EGCg and EGC by an HPLC system and the effects of catalase and SOD on cytotoxicity of these compounds by a colony-formation assay with cultured mammalian cells. Its purpose was to clarify the mechanisms of H_2O_2 formation in aqueous solutions of catechins, and to show examples of results affected by the formation of superoxide and H_2O_2 .

Materials and Methods

Reagents Sodium sulfate (Na_2SO_4), ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt (EDTA, 2Na) and catalase from bovine liver (5000–13000 units/mg) were purchased from Wako (Osaka). Copper/zinc superoxide dismutase from bovine erythrocytes (SOD) was obtained from Sigma Chemical Co. (St. Louis, MO). H_2O_2 (31% w/v) from Mitsubishi Gas Chemical Co., Ltd. (Tokyo) was freshly diluted prior to preparation of the standards. Sep-Pak Plus C18 cartridges (1 ml), short reverse-phase columns, were purchased from Millipore Corporation (Milford, MA), and were successively conditioned with 2 ml of acetonitrile and 4 ml of water before use. All other chemicals were of reagent grade and were used without further purification. All buffers were prepared with distilled water treated with a Milli-Q-Lab system from Japan Millipore, Ltd. (Tokyo).

Standard conditions of catechin incubation An aqueous catechin solution (500 μM) was prepared in 25 mM phosphate buffer (pH 7.0). Four milliliters of the solution in a lidless test tube was incubated in a water bath for 60 min at 37°C. After

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Abbreviations: EC, epicatechin; ECD, electrochemical detector; ECg, epicatechin gallate; EGC, epigallocatechin; EGCg, epigallocatechin gallate; FBS, fetal bovine serum; HBS, HEPES-buffered saline; SOD, superoxide dismutase

immediate cooling to 0°C, the solution was passed through a Sep-Pak C18 cartridge. The first 1 ml of effluent was discarded and the rest of the effluent (3 ml) was collected in a test tube and preserved at 0°C until H₂O₂ measurement by HPLC.

H₂O₂ measurement by HPLC The concentration of H₂O₂ formed in aqueous solutions of catechins was determined by HPLC equipped with an electrochemical detector (ECD). A method for separation of H₂O₂ by HPLC and its detection by ECD was developed in our laboratory (Takahashi *et al.*, 1999). Briefly, the system consisted of an HPLC column packed with a cation-exchange resin gel (Shodex Ionpak KS-801, 150 mm×8 mm *i.d.*; Showa Denko, Tokyo), two HPLC pumps (Jasco PU-980; Japan Spectroscopic, Tokyo) and an ECD (BAS LC-4C, West Lafayette, Indiana). The electrolyte was prepared with Na₂SO₄ (50 mM) and EDTA, 2Na (10 μM). The flow rate of pure water from the first pump through a column was 1.0 ml/min and that of the electrolyte from the second pump was 0.25 ml/min. ECD was equipped with a Pt electrode as a working electrode and an Ag/AgCl electrode as a reference electrode. The peak areas were calculated with a Chromatocoder 21J (System Instruments, Tokyo). The amount of H₂O₂ was determined with the aid of a calibration curve prepared with standards of H₂O₂. These standards were prepared daily with the phosphate buffer. Four

independent experiments at each measuring point with the same catechin solutions were carried out, and the results were expressed as the means and standard deviations (SD).

Colony formation assay Cytotoxic effects of catechins were assessed by a colony-formation assay. Chinese hamster lung fibroblast V79 cells were seeded in 60-mm petri dishes (100 cells/dish) and incubated in 5 ml of a minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ in air at 37°C for 4 h. After changing the medium to 5 ml of HEPES-buffered saline (HBS) containing the indicated concentration of each catechin, the cells were incubated for 30 min. After culture in MEM supplemented with 10% heat-inactivated FBS for 5 days, the colonies were fixed with methanol and stained with Giemsa's solution and their number was counted. The survival rate (% of control) was calculated by dividing the number of colonies of the cells treated with a catechin by that of the untreated control cells. Each result is expressed as the means and SD of four separately treated cultures. The data were analyzed by Student's *t*-test in some cases.

Measurement of catechins by HPLC The concentration of a catechin in the medium under the same conditions in the colony formation assay described above was measured by HPLC.

Fig. 1. Structures of the four catechins and the related compounds.

Table 1. Concentration of H₂O₂ formed in aqueous solutions of catechins and their related compounds.

	Concentration of H ₂ O ₂ (μM)
EC	21±3
ECg	0±0
EGC	179±5
EGCg	191±13
Pyrogallol	391±4
Gallic Acid	28±4
Gallic Acid Methyl Ester	4±1

An aqueous solution of each compound (500 μM) was prepared in 25 mM phosphate buffer (pH 7.0). After incubation for 60 min at 37°C, the concentration of H₂O₂ in the solution was measured by the HPLC-ECD system.

HBS (5 ml) containing each catechin (100 μM) was incubated in a dish at 37°C for 30 min, after which the amount of catechin in the medium was measured by HPLC with a UV detector using a Capcell Pak C18 UG120 column (Shiseido Co., Ltd., Tokyo). The mobile phase was 10% acetonitrile for EGC, 15% acetonitrile for EC and EGCg, and 18% acetonitrile for ECg with water in the presence of 0.1% trifluoroacetic acid and detection wavelength was 280 nm.

Results

Relationship between the chemical structure and the amount of H₂O₂ The concentration of H₂O₂ formed in aqueous solutions of catechins and their related compounds depicted in Fig. 1 was determined by the HPLC system (Table 1). After incubation for 60 min, the gallo catechins produced a higher amount of H₂O₂ than the catechol-type catechins, namely EC and ECg. These results suggest that the galloyl moiety in the B-ring contributed to the H₂O₂ formation. Since ECg and EGCg have a galloyl moiety, which also has three hydroxyl groups like the galloyl moiety, these compounds were expected to produce a high amount of H₂O₂.

Fig. 2. Dose dependency of H₂O₂ formation in aqueous EGCg solutions. Aqueous solutions of EGCg in the indicated concentrations were prepared in 25 mM phosphate buffer (pH 7.0). After incubation for 60 min at 37°C, the concentration of H₂O₂ in the solutions was measured by the HPLC-ECD system.

The results of EC and EGC compared with the results of ECg and EGCg, respectively, implied that the presence of the galloyl moiety does not significantly contribute to the formation of H₂O₂. To confirm this concept, we measured the concentration of H₂O₂ formed from the model compounds under the same incubation conditions used for tea catechins (Table 1). Pyrogallol is the simplest model of the galloyl moiety. Gallic acid and its ester, methyl gallate, are models of the galloyl moiety. Pyrogallol produced the highest amount of H₂O₂ among the compounds investigated in the present study, while the amounts produced by gallic acid and methyl gallate were lower. The low amount of

Fig. 3. Time dependency of H₂O₂ formation in aqueous EGCg solutions. Aqueous solutions of EGCg (500 μM) were prepared in 25 mM phosphate buffer (pH 7.0). After incubation at 37°C for the indicated incubation times, the concentration of H₂O₂ in the solutions was measured by the HPLC-ECD system. **Fig. 4.** pH dependency of H₂O₂ formation in aqueous EGCg solutions. Aqueous solutions of EGCg (500 μM) were prepared in 25 mM phosphate buffer with the indicated pHs. After incubation at 37°C for 60 min, the concentration of H₂O₂ in the solutions was measured by the HPLC-ECD system.

H₂O₂ in the aqueous solutions of ECg and methyl gallate indicates that the galloyl moiety in these compounds did not greatly contribute to the formation of H₂O₂.

Effects of the incubation conditions In the following experiments we investigated the formation of H₂O₂ during oxidation of EGCg, because the EGCg content in green tea infusions is usually the highest among the four catechins. The amount of H₂O₂ formed in the EGCg solution under the standard conditions

Fig. 5. Temperature dependency of H₂O₂ formation in aqueous EGCg solutions. Aqueous solutions of EGCg (500 μM) were prepared in 25 mM phosphate buffer (pH 7.0). After incubation for 8 min at the indicated temperatures, the concentration of H₂O₂ in the solutions was measured by the HPLC-ECD system.

Fig. 6. Effects of various amounts of SOD on H₂O₂ formation in aqueous solutions of gallic catechins. Aqueous solutions of the gallic catechins (500 μM) were prepared in 25 mM phosphate buffer (pH 7.0) with the indicated amount of SOD. After incubation at 37°C for 60 min, the concentration of H₂O₂ in the solutions was measured by the HPLC-ECD system. ● EGCg; ○ EGC.

varied in each experiment, but was around 180 to 220 μM. Figure 2 shows that the measured amount of H₂O₂ was almost 40% of the amount of EGCg added at these concentrations. H₂O₂ increased almost linearly with the incubation time until 60 min and then increased gradually (Fig. 3). Figure 4 shows the pH dependency of H₂O₂ formation in 25 mM sodium phosphate buffer. H₂O₂ was detected above pH 5.8 and its concentration steeply increased with pH up to pH 8.0. The effect of incubation temperature on the amount of H₂O₂ was investigated. After incubation of EGCg in 25 mM sodium phosphate buffer (pH 7.0) for 8 min, the amount of H₂O₂ was 16 μM at 37°C, and increased with rising temperature up to 77°C (Fig. 5), which is close to the temperature of tea infusions. We next investigated the effects of SOD on the amount of H₂O₂. SOD dose-dependently inhibited H₂O₂ formation in the solution of both EGCg and EGC (Fig. 6). Inactivated SOD showed no inhibitory effects (data not shown), indicating that the inhibitory effects of SOD were due to its enzymatic activity.

Cytotoxic effects of catechins Figure 7 shows the effects of various doses of the catechins on the survival rate of V79 cells. At both 100 μM and 500 μM, the order of toxicity was EGC ≅ EGCg ≫ ECg; EC showed no cytotoxicity at these concentrations. These results indicate that the gallic catechins showed higher cytotoxicity than the catechol-type catechins. To verify that the difference of the cytotoxic activities between the gallic catechins and the catechol-type catechins is ascribable to the H₂O₂ formation, we investigated the effects of catalase and SOD in cytotoxicity of the gallic catechins. The results of experiment 1 (Table 2) show that catalase almost abolished the cytotoxic effects of EGCg and EGC. This confirms that the cytotoxic effects were ascribable to the formation of H₂O₂. The result of experiment 2

Fig. 7. Effects of four catechins on the survival rate of Chinese hamster lung V79 cells. V79 cells (100 cells/dish) were incubated in 5 ml of HBS with the indicated concentration of each catechin for 30 min. The number of colonies was counted after culturing in MEM supplemented with 10% FBS for 5 days. The survival rate (% of control) was calculated by dividing the number of colonies in each catechin medium by that in the control medium. The results are expressed as the mean and standard deviation of four separately treated cultures. ● EGC; ▲ EGCg; ○ EC; △ ECg.

Table 2. Effects of catalase and SOD on the survival rate of Chinese hamster lung V79 cells treated with EGCg or EGC.

	Survival of cells (% of control)	
	+catalase	-catalase
Exp.1		
EGCg	102±37*	8±3
EGC	91±13*	1±1
Exp.2		
EGCg	+SOD	-SOD
EGCg	62±17*	21±4
EGC	74±9*	11±3

V79 cells (100 cells/dish) were incubated in 5 ml of HBS with each catechin (100 μ M) for 30 min in the presence or absence of catalase (2 μ g/dish) in experiment 1 and SOD (50 units/dish) in experiment 2. These experiments were carried out on separate days. The number of colonies was counted after culturing in MEM supplemented with 10% FBS for 5 days. The survival rate (% of control) was calculated by dividing the number of colonies in each catechin medium by that in the control medium. Results are expressed as the mean and standard deviation of four separately treated cultures. *Significantly different from the corresponding value in the absence of the respective enzyme at $p < 0.05$ (Students' *t*-test).

in Table 2 shows that SOD significantly inhibited the cytotoxicity of EGCg and EGC. Under the same conditions, the gallo catechins were more unstable than the catechol-type catechins and SOD inhibited decomposition of the gallo catechins (Table 3).

Discussion

Formation of H₂O₂ during oxidation of tea catechins in aqueous solutions has been previously reported. H₂O₂ was formed in aqueous (+)-catechin solutions during the aerobic heating at 80°C (Nakayama *et al.*, 1995). The effects of pH and incubation time on the amount of H₂O₂ were similar to our present results with EGCg solutions at 37°C. Miura *et al.* (1998) detected H₂O₂ in EC and (+)-catechin solutions incubated at 21°C. Hoshino *et al.* (1999) reported that H₂O₂ was formed in a buffer containing EGC in the presence of Cu (II). Long *et al.* (2000) found that H₂O₂ was time-dependently generated in various cell-culture media after incubation of (+)-catechin, EGC, EGCg and gallic acid. Among these compounds EGCg and EGC produced a relatively high amount of H₂O₂. Yang *et al.* (2000) reported that apoptosis of a human bronchial cell line induced by EGCg and EGC was ascribable to H₂O₂ production in the cells. These results indicate that a relatively high amount of H₂O₂ was formed in the aqueous solutions of the gallo catechins such as EGCg and EGC. However, they did not refer to the role of superoxide anion in the formation of H₂O₂.

We have established a method to separate H₂O₂ by HPLC and to quantify its amount by ECD (Takahashi *et al.*, 1999). With this highly selective method, we succeeded in the measurement of H₂O₂ in coffee and aqueous ascorbic acid solutions. In the present study, we confirmed the formation of H₂O₂ in aqueous solutions of various catechins by this HPLC-ECD system and elucidated the relationship between the structure and the amount of H₂O₂ as follows. Tea catechins possess two kinds of benzene rings with three hydroxyl groups. The first one is a gallyl moiety in the B-ring, which is generally represented as "G" in the abbreviations of catechins. The second one is a galloyl moiety, which is represented as "g" in the abbreviations of gallic acid esters of catechins. Both moieties are thought to have reducing ability and antioxidant activity. Thus, it is reasonable to suppose that they are easily oxidized and that H₂O₂ is formed during oxidation of these compounds, but there has been no report on which moiety

Table 3. Effects of SOD on the amount of catechin in HBS after incubation.

	Concentration of catechin (μ M)	
	-SOD	+SOD
EGCg	15.0±1.5	59.4±5.7
EGC	13.8±3.5	58.7±4.8
ECg	70.2±0.7	64.6±3.6
EC	80.7±2.0	69.2±5.0

HBS (5 ml) containing each catechin (100 μ M) was incubated in the absence or presence of SOD (50 unit) at 37°C for 30 min. After incubation, the amount of catechin in HBS was measured by HPLC. The results are expressed as the mean and standard deviation of four separately treated samples.

has the greater role in the H₂O₂ formation. The concentration of H₂O₂ formed in the aqueous solutions of the four catechins listed in Table 1 is as follows: EC>ECg, EGC=EGCg, EGC>>EC, EGCg>>ECg. This indicates that the gallyl moiety in EGC and EGCg contributed significantly to the formation of H₂O₂, but that the galloyl moiety in ECg and EGCg did not. The much higher amount of H₂O₂ formed in the aqueous solution of pyrogallol than that in the solution of methyl gallate (Table 1) supports the idea that the addition of an ester structure to the pyrogallol ring lowers the ability to produce H₂O₂. The substituents such as carboxylic acid and its esters are electron-withdrawing groups. Reduction of electron density of the benzene ring in the esters might result in less electron-donating ability of the galloyl moiety than that of the gallyl moiety.

SOD dose-dependently inhibited H₂O₂ formation in the aqueous solutions of EGCg and EGC (Fig. 6). This result seems contradictory to the function of SOD, because H₂O₂ is a product of its enzyme reaction. We solved this problem by postulating a chain reaction with O₂^{•-} as the chain carrier (Fig. 8), as already proposed for the oxidation of (+)-catechin (Nakayama *et al.*, 1995). In this reaction, O₂^{•-} instead of O₂ initiates the oxidation of a gallyl moiety, resulting in the formation of a semiquinone-type radical and H₂O₂ (reaction (1)). The formed radical rapidly reacts with O₂, resulting in the formation of various oxidation products and O₂^{•-} (reaction (2)). The produced O₂^{•-}, in turn, oxidizes another molecule with the gallyl moiety; thus, the chain reaction continues. The sum of reactions (1) and (2) is the observed overall reaction where no O₂^{•-} appears. The rate constant of the spontaneous dismutation of superoxide is 5×10⁵ M⁻¹ s⁻¹, while the rate constant of enzymatic dismutation of superoxide by SOD is 2×10⁹ M⁻¹ s⁻¹ (Ogawa & Asada, 1996). This means that the latter reaction is almost diffusion controlled and the concentration of O₂^{•-} can be almost 10³ times lower than that in the absence of SOD. Thus, SOD inhibits reaction (1) by lowering the concentration of O₂^{•-} and consequently inhibits the formation of H₂O₂ in the overall reaction by reducing the turnover number of the chain reaction. Inhibition of oxidation of the gallo catechins (Table 3) by SOD supports this mechanisms. Inhibition of H₂O₂ formation by SOD during the oxidation of various compounds has been experimentally proved (Cadenas *et al.*, 1988; Nakayama *et al.*, 1989; Bandy *et al.*, 1990; Nakayama *et al.*, 1992a; Nakayama *et al.*, 1997; Hiramoto *et al.*, 2001). Furthermore, inhibition of pyrogallol oxidation by SOD has been used as an assay for SOD (Marklund & Marklund, 1974). Thus, inhibition of H₂O₂ formation by SOD during oxidation is not peculiar to the

Fig. 8. Proposed mechanism of oxidation of gallo catechins. Only the gallyl moiety in the B-ring is depicted.

gallo catechins.

The order of the affinity of the catechins for lipid bilayers was as follows: ECg>EGCg>EC>EGC (Hashimoto *et al.*, 1999; Kajiya *et al.*, 2001b). The orders of biological activities studied by *in vitro* experiments reflected this order to some degree in many cases. Figure 7, however, shows that the order of the cytotoxic activity under our experimental conditions with V79 cells was EGC=EGCg>>ECg>EC, which was different from the order of the affinity for lipid bilayers described above. The higher cytotoxicity of the gallo catechins than the catechol-type catechins suggested that the cytotoxic activity was partly ascribed to the formation of H₂O₂. This idea was confirmed by the inhibitory effects of catalase on the cytotoxicity (Table 2). The amount of H₂O₂ formed during incubation of EGCg (100 µM) for 30 min was nearly equal to that of H₂O₂ formed during incubation of EC (500 µM) for 60 min (Fig. 2 and Table 1). Cytotoxicity of EGCg (100 µM) was, however, much higher than that of EC (500 µM) (Fig. 7). In addition, no H₂O₂ was formed during incubation of ECg (500 µM) for 60 min (Table 1), yet ECg showed higher cytotoxicity than EC (Fig. 7). These results suggest that H₂O₂ formation is not the only factor governing the cytotoxicity of catechins. As described above, higher affinity of ECg and EGCg for the lipid bilayers than of EC might also contribute to their cytotoxicity to some degree. The inhibitory effect of SOD does not indicate that O₂⁻ directly participates in the cytotoxicity of the gallo catechins, because O₂⁻ formed in a medium with an O₂⁻ producing system such as hypoxanthine-xanthine oxidase does not enter cells, while not SOD but catalase in the medium inhibits the cytotoxicity. Since SOD inhibited decomposition of the gallo catechins (Table 3) and lowered the amount of H₂O₂ formed in their aqueous solutions (Fig. 6), the inhibitory effects of SOD on the cytotoxicity of the gallo catechins should be ascribed to the inhibition of H₂O₂ formation during their oxidation process.

Long *et al.* (2000) measured H₂O₂ formed in various cell culture media containing polyphenols and claimed that the effects of H₂O₂ should be considered when examining the biological activ-

ities of polyphenols by *in vitro* experiments. The results of the present study and those reported by Yang *et al.* (2000) show that formation of H₂O₂ in aqueous solutions can affect the results of the *in vitro* experiments with tea catechins. The cytotoxicity of the four flavonols determined by a colony-formation assay was in the order of myricetin>galangin>kaempferol>quercetin (Kajiya *et al.*, 2001a). Apart from myricetin, the order of cytotoxicity was the same as that of lipophilicity. Only the cytotoxicity of myricetin was inhibited by catalase. EGCg, EGC and myricetin possess a common gallyl moiety. Thus, participation of H₂O₂ in the biological activities studied *in vitro* seems to be characteristic of the compounds with a gallyl moiety. A small amount of H₂O₂ was detected in aqueous solutions of polyphenols with a catechol moiety or a galloyl moiety, *e.g.* (+)-catechin, quercetin and methyl gallate in this and previous papers (Long *et al.*, 2000; Nakayama *et al.*, 1995; Miura *et al.*, 1998; Kajiya *et al.*, 2001), but these compounds inhibited the cytotoxic effects of H₂O₂ in the cells (Nakayama *et al.*, 1992b; Nakayama *et al.*, 1993). We assume that higher amounts of these compounds were incorporated into the cells during incubation and less H₂O₂ was produced in the medium than the compounds with a gallyl moiety. Consequently, the antioxidant effects of the former compounds were supposed to overcome the pro-oxidant effects of H₂O₂. H₂O₂ would not be formed from tea catechins in our body. Some tea infusions, however, contained appreciable amounts of H₂O₂ (data not shown). The results of the present study indicate that the amount of H₂O₂ in tea infusions depends on various factors such as the composition of catechins, standing time, pH, and temperature. Consideration of the effects of H₂O₂ might be necessary to estimate the biological activities of tea infusions, especially in our mouth and digestive tract.

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