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# Prevention of the deterioration of polyphenol-rich beverages

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**Abstract**

Only a small amount of $H_2O_2$ was detected in beverages, such as tea or coffee, immediately after opening caps of bottles, but $H_2O_2$ was gradually produced in the beverages after opening the caps, i.e. exposure to air. The beverages with high concentrations of polyphenols showed high 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities, but produced relatively high concentrations of $H_2O_2$ when they were exposed to air, possibly due to oxygen. The production of $H_2O_2$ increased both with duration of the exposure to air and rise in temperature. Since $H_2O_2$ is toxic, ways to prevent the deterioration of catechin-enriched green tea, i.e. $H_2O_2$ production, were studied. The addition of catalase, which is an enzyme decomposing $H_2O_2$, reduced the $H_2O_2$ concentration, but it was inactivated at a high temperature. The addition of L-cysteine or glutathione (reduced form), with a thiol residue, reduced the $H_2O_2$ concentration. Addition of citric acid, malic acid, succinic acid, fumaric acid, L-ascorbic acid, L-glutamic acid and L-aspartic acid also reduced it, possibly because they lower the pH of the tea.


*Keywords:* Antioxidant; Catechin; Hydrogen peroxide; Polyphenol; Tea

## 1. Introduction

Polyphenols are present in various beverages and known to work as antioxidants (Bravo, 1998; Ina, Sakata, Tomita, & Isemura, 2002). Antioxidants are very important for human health, since the production of reactive oxygen species is thought to be a significant cause of aging and carcinogenesis (Beckman & Ames, 1997; Lambert & Yang, 2003). Actually, polyphenols can act as free radical-scavengers, quenching hydroxyl radicals (‘OH) or superoxide anion radicals ($O_2^-$) (Hanasaki, Ogawa, & Fukui, 1994; Sichel, Corsaro, Scalia, Di Bilio, & Bonomo, 1991). Recent epidemiological studies have shown that flavonoid-rich beverages, such as red wine, have beneficial effects for carcinogenesis and cardiovascular diseases (Soleas, Diamandis, & Goldberg, 1997).

In contrast to the beneficial effects of polyphenols, production of hydrogen peroxide ($H_2O_2$) from polyphenols, such as catechin derivatives, has been recently reported (Arakawa, Maeda, Okubo, & Shimamu, 2004; Cao, Sofic, & Prior, 1997; Long, Lan, Hsuan, & Halliwell, 1999; Nakayama, Ichiba, Kuwabara, Kajiya, & Kumazawa, 2002). Reportedly, $H_2O_2$ was produced from polyphenol-rich beverages under quasi-physiological conditions and increased with the incubation time (Akagawa, Shigemitsu, & Suyama, 2003; Chai, Long, & Halliwell, 2003). It is known that $H_2O_2$ is toxic and induces cell death in vitro (Aoshima, Hossain, Tanaka, & Wen, 2004; Aoshima, Kadoya, Taniguchi, Satoh, & Hatanaka, 1999; Fuchs, Baier-Bitterlich, Wede, & Wachter, 1997; Whittermore, Loo, & Cotman, 1994). It has been reported that some polyphenols promote oxidative damage to DNA, lipids and deoxyribose under certain conditions in vitro (Hayakawa, Kimura, Hoshino, & Ando, 1999; Hayakawa et al., 1997; Yamanaka, Oda, & Nagao, 1997). Various beverages are sold in vending machines in Japan. Oolong tea, in bottles with sugar, is also very popular in China. The beverages are often stored in vending machines at high temperatures for several days. Moreover, catechin-enriched

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green teas are very popular in Japan because of their antioxidative and dietary effects. Therefore, in this research, we measured the $H_2O_2$ concentrations in these beverages. The total polyphenol (Ough & Amerine, 1988) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities (Blois, 1958) of beverages were also measured to find the relationships among three measurements.

It is desirable to prevent $H_2O_2$ production after the cap of beverage bottles is opened, since we sometimes drink beverages over several hours after opening. So, we added various compounds to catechin-enriched green tea and examined their effects on $H_2O_2$ production to find easy and safe methods for $H_2O_2$ reduction in beverages.

## 2. Materials and methods

### 2.1. Chemicals and samples

Xylenol orange, butylated hydroxytoluene (BHT), methanol, glutathione (reduced form), DPPH and bovine liver catalase (11,500 units/mg; 1 unit of catalase decomposes 1 m mole $H_2O_2$ $min^{-1}$ at 25 °C and pH 7.0 in the presence of 0.2% $H_2O_2$) were purchased from Wako Pure Chemical Industry, Ltd., Osaka, Japan. Gallic acid, citric acid, malic acid, succinic acid, fumaric acid, L-aspartic acid, L-cysteine and ammonium ferrous sulfate were purchased from Nacalai Tesque, Kyoto, Japan. Folin and Ciocalteu's phenol reagent and L-ascorbic acid were purchased from Katayama Chemical Industry, Osaka, Japan. All chemicals used were of guaranteed reagent quality. Beverages which were extracted in aqueous solution and stored in polyethylene terephthalate (PET) bottles or cans, were purchased from a local market in Yamaguchi, Japan. For comparison, we bought the following green teas, produced by different companies; Green tea 1 (GT1): presence of 600 mg/l of polyphenols (mainly catechin derivatives) and 139 mg/l of caffeine, GT2: added by green tea powder to GT, GT3: added by 114 mg/l flavandienol (polyphenol prepared from pine trees) to GT, GT4: presence of 1.57 g/l of catechin derivatives and 232 mg/l of caffeine. The teas had an undescribed amount of L-ascorbic acid added. Teas were contained in PET bottles and coffees were filled in cans.

### 2.2. Determination of $H_2O_2$ by the ferrous ion oxidation-xylenol orange (FOX) assay

The concentration of $H_2O_2$ was measured as described (Akagawa et al., 2003; Long et al., 1999). FOX reagent was prepared by adding one volume of Reagent 1 to nine volumes of Reagent 2, where Reagent 1 was 4.4 mM 2,6-di-*t*-butyl-4-methylphenol (BHT) in methanol and Reagent 2 was 1 mM xylenol orange plus 2.56 mM ammonium ferrous sulfate in 250 mM $H_2SO_4$. The samples (100 μl) in which the $H_2O_2$ concentrations were measured, were added to the FOX reagent (3 ml) and vortexed for 5 s. After incubation for 30 min at room temperature and centrifugation, the absorbance at 560 nm was measured using a spectrometer (Hitachi U-2000A). The FOX assay was calibrated using a standard $H_2O_2$ solution whose concentration was estimated by using a molar extinction coefficient of 43 $M^{-1}$ $cm^{-1}$ at the 240 nm absorbance wavelength of $H_2O_2$.

### 2.3. Determination of total polyphenol with the Folin assay

Total polyphenol contents in beverages were analyzed by the Folin assay (Ough & Amerine, 1988). For the analyses, gallic acid was used as the standard and the polyphenol concentrations in beverages were expressed (mM) as gallic acid equivalents. One ml of beverages or liquors diluted 20 times with deionized water was mixed with 1 ml of Folin–Ciocalteu's reagent. After vortexing for 5 s, 1 ml of 10% (w/w) sodium carbonate aqueous solution was added to the mixture. The mixture was incubated at room temperature for 1 h, followed by colorimetric measurement at 700 nm.

### 2.4. Measurement of DPPH radical-scavenging activity

The reaction mixture, (total volume, 3 ml) consisting of 0.5 ml of 0.5 M acetic acid buffer solution at pH 5.5, 1 ml of 0.2 mM DPPH in ethanol and 1.5 ml of 50% (v/v) ethanol aqueous solution, with or without beverages, was shaken vigorously with various samples (Blois, 1958; Aoshima, Tsunoue, Koda, & Kiso, 2004). The final concentrations of the beverages in the reaction mixture were 2.5 ml/l. After incubation at room temperature for 30 min, the remaining DPPH was determined by the absorbance at 517 nm, and the radical-scavenging activity of each sample was expressed using the ratio of the absorption decrease of DPPH (%) relative to the control DPPH solution (100%) in the absence of the sample. When the diluted sample itself had a 517 nm-absorption more than 1% of the control absorption, it was subtracted from the 517 nm-absorption of the sample reaction mixture. That is, the radical-scavenging activity (%) $= 100(A - B)/A$, where $A$ and $B$ were the 517 nm-absorption of the control and the corrected absorption of the sample reaction mixture.

### 2.5. Reduction of $H_2O_2$ in catechin-enriched green tea by L-cysteine, glutathione and catalase

L-Cysteine, glutathione (reduced form) and catalase were dissolved in 100 mM phosphate buffer at pH 7.4. These solutions were mixed with an equal volume of catechin-enriched green tea (GT4) and incubated. Then, the amount of $H_2O_2$ was measured using the Fox assay. When the dose effect of the compounds on the $H_2O_2$ production was examined, the mixture was incubated for 24 h at 37 °C. When the effect of temperature on the $H_2O_2$ production was examined, the mixture was incubated for 24 h. When the effect of incubation time on the $H_2O_2$ production was examined, the mixture was incubated for various periods at 37 °C. The concentrations of L-cysteine, glutathione

and catalase in the mixtures were 14 mM, 6 mM and 10 units/ml respectively, for both experiments.

### 2.6. Reduction of $H_2O_2$ in GT4 by acidic amino acids and organic acids

Citric acid, malic acid, succinic acid, fumaric acid, L-ascorbic acid, L-glutamic acid and L-aspartic acid were each dissolved in deionized water. When their effects on the $H_2O_2$ production were examined at pH 7.4, they were dissolved in 100 mM phosphate buffer at pH 7.4. One volume of these samples was added to nine volumes of GT4. This mixture was mixed with an equal volume of deionized water and incubated for 24 h at 60 °C. Then, the concentration of $H_2O_2$ was measured with the FOX assay.

## 3. Results

The concentrations of $H_2O_2$ in beverages in PET bottles (teas) or cans (coffees) were measured by the FOX assay. All examined beverages contained very small amounts of $H_2O_2$ just after opening the caps of PET bottles or cans (Fig. 1). However, the $H_2O_2$ in beverages increased several-fold when they were incubated in a plastic tube for 24 h at 25 °C. Since much $H_2O_2$ was produced in polyphenol-enriched green tea, i.e. GT4 and black tea, we measured the $H_2O_2$ concentrations of these beverages incubated in a plastic tube for various time periods at 25 °C and found that generation of $H_2O_2$ increased with the incubation time up to 12 h and reached a plateau (data not shown).

Table 1
Total polyphenol and DPPH radical-scavenging activity in beverages

| Beverages | Total polyphenol (mM) | DPPH-scavenging activity (%) |
|---|---|---|
| GT1 | 3.29 ± 0.03 | 62.6 ± 0.8 |
| GT2 | 3.38 ± 0.01 | 64.5 ± 1.5 |
| GT3 | 3.12 ± 0.01 | 61.8 ± 1.5 |
| GT4 | 9.81 ± 0.09 | 90.0 ± 0.2 |
| Oolong tea | 1.61 ± 0.00 | 32.3 ± 1.1 |
| Black tea | 3.22 ± 0.01 | 52.8 ± 1.2 |
| Coffee | 4.22 ± 0.01 | 64.2 ± 1.5 |
| 20 μM Cys | Not measured | 14.7 ± 1.1 |
| 100 μM Cys | Not measured | 89.4 ± 2.5 |

Concentration of total polyphenol was expressed as mM gallic acid equivalents. Data are means ± SD, $n = 3$. $P < 0.05$ between the control value and the beverage ones, with Student's $t$-test.

To examine the relationship among $H_2O_2$ production, total polyphenol and antioxidative activity in beverages, we measured both total polyphenol and the DPPH radical-scavenging activities of beverages (Table 1). The correlation factor between the DPPH radical-scavenging activities and the total polyphenol concentrations was estimated to be 0.870, and that between the $H_2O_2$ concentrations and total polyphenol concentrations was estimated to be 0.864 (Fig. 1 and Table 1).

The procedures to reduce $H_2O_2$ were examined by using the catechin-enriched green tea (GT4), which produces

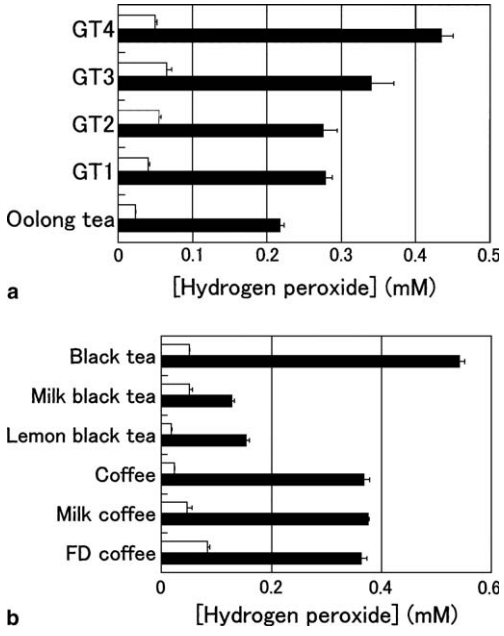


Fig. 1. The concentration of $H_2O_2$ in beverages: (a) green teas and oolong tea; (b) black teas and coffees. The hot beverages kept in polyethylene terephthalate (PET) bottles or cans were purchased from vending machines. The concentrations of $H_2O_2$ in these beverages were measured with the FOX assay, just after opening the caps of the PET bottles (open bars) and after incubation in a plastic tube for 24 h at 25 °C (closed bars). The concentration of $H_2O_2$ in an instant (frozen dried; FD) coffee just after preparation was also measured and is shown in (b). Data are means ± SD, $n = 3$. $P < 0.05$ by Student's $t$-test.

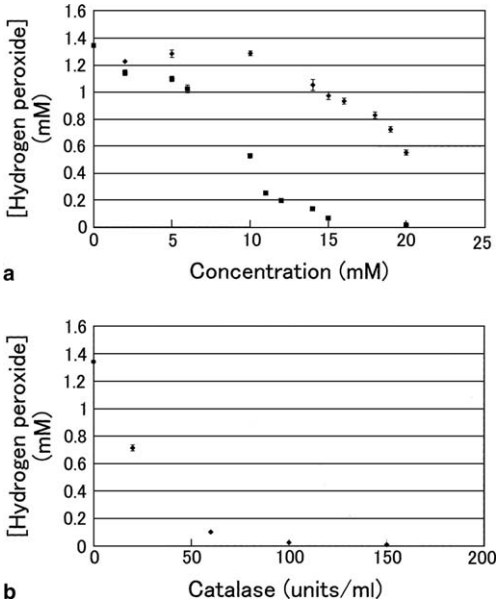


Fig. 2. Dose-dependent effects of: (a) L-cysteine (♦) and glutathione (■), and (b) catalase on the reduction of $H_2O_2$ in GT4. Various concentrations of the compounds were dissolved in 0.1 M phosphate buffer at pH 7.4 and mixed with an equal volume of GT4. The mixtures were incubated for 24 h at 37 °C and their concentrations were measured using the FOX assay. Data are means ± SD, $n = 3$.

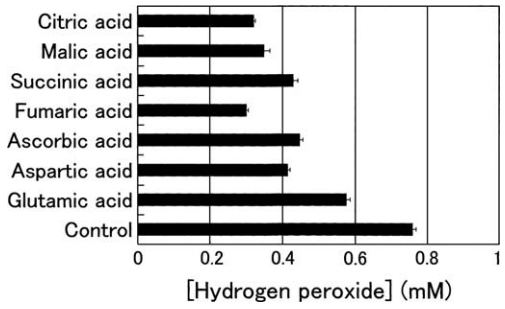


Fig. 3. The protective effects of acidic amino acids and organic acids at 1 mM against $H_2O_2$ production in GT4. One volume of deionized water with various compounds was mixed with an equal volume of GT4. These mixtures were incubated for 24 h at 60 °C and the $H_2O_2$ concentrations were measured by FOX assay. Data are means ± SD, $n = 3$. $P < 0.05$ between the control value and the values in the presence of compounds, using Student's *t*-test.

much $H_2O_2$. First, in phosphate buffer at pH 7.4, we examined L-cysteine and glutathione (reduced form) with thiol residue, and catalase, which decomposes $H_2O_2$, since $H_2O_2$ production in the buffer at pH 7.4 was more than in deionized water (Akagawa et al., 2003). As shown in Fig. 2, these compounds reduced the $H_2O_2$ in this green tea dose-dependently. Then, we examined both time and temperature dependence in the production of $H_2O_2$ (data not shown). The protective activities of L-cysteine and glutathione for $H_2O_2$ production in GT4 decreased with both the incubation time and the temperature rise, possibly because they increased the $H_2O_2$ production. On the other hand, catalase is very effective in the reduction of $H_2O_2$, but becomes ineffective at high temperatures (60 °C) because of its inactivation.

Second, since it was reported that the $H_2O_2$ production in beverages is dependent on pH (Akagawa et al., 2003), we examined the amino acids and organic acids that are present in the human body and that lower the pH of the beverages, and found that they lowered pH (about pH 3) of GT4 detected by pH test paper and also reduced the $H_2O_2$ production in the beverage (Fig. 3). As expected, however, these compounds lost their protective effect against $H_2O_2$ production in the phosphate buffer at pH 7.4 (data not shown).

## 4. Discussion

Polyphenols in beverages are noticeable and popular because of their beneficial physiological effects on human health. Catechin and its derivatives, found in abundance in green tea, possess physiological effects, including antioxidative, dietary and bactericidal action, as well as antitumor activity (Hara, 2001; Ina et al., 2002). Chlorogenic acid in coffee is also reported to have antitumor activity. However, the production of hydrogen peroxide ($H_2O_2$) from polyphenols was reported and its mechanism was recently proposed (Akagawa et al., 2003; Chai et al., 2003).

In Japan, aqueous extracts of green tea, black tea, oolong tea and coffee in PET bottles or cans are sold everywhere in vending machines and are often kept hot for several days. Some green teas are fortified with polyphenols, such as catechins or flavandienol, to increase their physiological activity. So, we measured the $H_2O_2$ and total polyphenol concentrations and the DPPH radical-scavenging activities of beverages and found their close relationships. Fortunately, beverages in PET bottles or cans contained very small amounts of $H_2O_2$ just after the caps were detached and were proven to be almost free from $H_2O_2$ toxicity even if they had been stored in vending machines for several days at high temperatures. So beverages kept in bottles or cans are safe from $H_2O_2$ production when they are drunk soon after opening the cap of the bottle. The beverage companies must have filled the bottles or cans with beverages without oxygen to prevent $H_2O_2$ production and added ascorbic acid (vitamin C) to teas as an antioxidant. Ascorbic acid generates $H_2O_2$ but suppresses its production when ascorbic acid is mixed with polyphenols (Wee, Long, Whiteman, & Halliwell, 2003).

After opening the caps and exposing to air, the $H_2O_2$ in the beverages increased after incubation for 24 h at 25 °C, as observed in beverages extracted from tea leaves or ground coffee beans in deionized water (Akagawa et al., 2003; Chai et al., 2003). Beverages with high concentrations of total polyphenols usually had high DPPH radical-scavenging activities, but produced large amounts of $H_2O_2$ (Table 1 and Fig. 1). The production of $H_2O_2$ increased with rising temperature. After opening the cap of the beverage bottle, the bottle should be kept at a low temperature, for example, in a refrigerator. In Japan, we have a proverb "Do not drink tea extracted the day before", which is thought to be due to the propagation of bacteria in tea. However, this may be true due to the production of $H_2O_2$.

The toxicity of $H_2O_2$ was measured in vitro. It causes apoptosis in cells through oxidative stress (Aoshima et al., 2004, 1999; Fuchs et al., 1997; Whittermore et al., 1994). Green tea or polyphenols in green tea also caused cell death in PC12 cells or bacteria (Chai et al., 2003; Arakawa et al., 2004). However, toxic side effects of polyphenol-rich beverages have not been reported in vivo until now, possibly for the following reasons. Only 5–8% of the total polyphenol in foods and beverages is taken into the body through the intestines. Since reactive oxygen species, such as $O_2^-$ or $H_2O_2$, are constantly produced during oxidative phosphorylation, cells have a ubiquitous protective system against oxidative stress, such as superoxide dismutase or catalase (Richter & Schweizer, 1997), although it was reported that coffee drinking increases urinary $H_2O_2$ levels (Long & Halliwell, 2000). The epithelial cells in the stomach and intestines are always reproduced, even when the superficial ones are led to death.

Though some polyphenols produce toxic $H_2O_2$, they also have a beneficial activity, i.e. an antioxidative one. Since some polyphenols cause cell death by perturbing the membrane structure (Aoshima et al., 2005; Hossain

et al., 2002), much attention should be paid to their cell toxicity when they are enriched in beverages. In future, it is necessary to clarify the beneficial (Duthie et al., 1998; Ina et al., 2002) and toxic (Fujita, Wakabayashi, Nagao, & Sugimura, 1985; Yang et al., 2000) effects of each polyphenol in vivo and to evaluate the uptake of each polyphenol for human health.

We sometimes drink beverages in large bottles over a few days, and sometimes make coffee and keep it in a percolator pot for several hours. By reducing only the toxicity, i.e. $H_2O_2$, we may exclusively receive the beneficial effects of polyphenols. So, we examined the ways by which $H_2O_2$ could be reduced in catechin-enriched tea under aerobic conditions. The addition of catalase reduced the $H_2O_2$ concentration very efficiently, but it was inactivated at a high temperature, 60 °C. The addition of L-cysteine or glutathione, with thiol residue, also reduced the $H_2O_2$ concentration. These compounds and the enzyme can increase the rate of $H_2O_2$ decomposition and reduce the $H_2O_2$ concentration in beverages. The protective effects of L-cysteine and glutathione were weak in phosphate buffer at pH 7.4 and at high temperatures, possibly because more $H_2O_2$ was produced at a higher pH and temperature. Moreover, they reduced their protective activity with incubation time, possibly because they changed from reduced forms to oxidized forms. The addition of citric acid, malic acid, succinic acid, fumaric acid, L-ascorbic acid, L-glutamic acid and L-aspartic acid also reduced it, possibly because they lower the pH of the beverages. Polyphenols are autoxidised at a higher rate as the pH value is increased, because the acid dissociation of the phenolic group has a stronger electron-donating capacity than the undissociated one (Mochizuki, Yamazaki, Kano, & Ikeda, 2002). Though we have not examined the applicability of these methods to other beverages, such as black tea, oolong tea or coffee, we think that these methods are able to reduce $H_2O_2$ production. These amino acids and organic acids are safe because they are present in our body or are often eaten. However, they may change the odour or the taste of the beverages. So, it is necessary to examine which compound is best for each beverage in the prevention of $H_2O_2$ production or to find new compounds that reduce the $H_2O_2$ production without affecting the beverage taste.

## 5. Conclusion

Though only a small amount of $H_2O_2$ was detected in beverages, such as tea or coffee, immediately after opening caps of bottles, $H_2O_2$ was gradually produced in beverages after opening the caps, i.e. exposure to air. The production of $H_2O_2$ showed close relationships with both total polyphenol concentration and DPPH radical-scavenging activities. Production of $H_2O_2$ was suppressed by addition of catalase, L-cysteine or glutathione, with thiol residue, and acidic compounds, such as citric acid, malic acid, succinic acid, fumaric acid, L-ascorbic acid, L-glutamic acid and L-aspartic.

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