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# Aqueous extract of citrus peel reduces production of hydrogen peroxide in catechin-enriched green tea

S. Ayabe, H. Aoshima *

*Department of Biology and Chemistry, Faculty of Science, Yamaguchi University, Yoshida, Yamaguchi 753-8512, Japan*

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**Abstract**

Hydrogen peroxide ($H_2O_2$) is gradually produced in bottle-packed beverages, including tea and coffee, after the cap has been opened, i.e, through exposure to air, though only a small amount of $H_2O_2$ is detected in the beverage immediately after the bottle is opened. Since, $H_2O_2$ is toxic, it is necessary to develop safe and simple ways of reducing its production in bottled beverages. The addition of an aqueous extract of citrus peel reduced the concentration of $H_2O_2$ in green tea. To characterise the active constituents in the citrus peel, the aqueous extract of the peel was fractionated using chloroform, ethyl acetate, and butanol, in that order, and subjected to gel chromatography. The active constituents in the citrus peel were water-soluble compounds of various molecular weights.


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

## 1. Introduction

Polyphenols are present in various beverages and are known to act 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; Rice-Evans, Miller, & Paganga, 1996). Polyphenols can act as free radical scavengers, quenching the hydroxyl radical (·OH) or superoxide anion radical ($O_2^-$) (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 on carcinogenesis and cardiovascular diseases (Soleas, Diamandis, & Goldberg, 1997).

In contrast to the beneficial effects of polyphenols, the production of hydrogen peroxide ($H_2O_2$) from polyphenols, such as catechin derivatives, has been reported (Arakawa, Maeda, Okubo, & Shimamura, 2004; Cao, Sofic, & Prior, 1997; Long, Lan, Hsuan, & Halliwell, 1999; Mochizuki, Yamazaki, Kano, & Ikeda, 2002; Nakayama, Ichiba, Kuwabara, Kajiya, & Kumazawa, 2002). $H_2O_2$ was produced from polyphenol-rich beverages under quasi-physiological conditions and accumulated with incubation time (Akagawa, Shigemitsu, & Suyama, 2003; Chai, Long, & Halliwell, 2003). $H_2O_2$ is toxic (Fujita, Wakabayashi, Nagao, & Sugimura, 1985) and induces cell death *in vitro* (Aoshima, Kadoya, Taniguchi, Satoh, & Hatanaka, 1999; Fuchs, Baier-Bitterlich, Wede, & Wachter, 1997; Whittermore, Loo, & Cotman, 1994). In the presence of transition metals, $H_2O_2$ can be reduced to generate ·OH via the Fenton reaction, which can attack cell membranes by setting off free radical chain reactions (Fuchs et al., 1997; Nakagawa et al., 2002). Such a reaction cascade results in extensive damage of cell membranes and other macromolecules such as proteins and DNA. The cell death may be induced through $H_2O_2$ receptors such as TRPM2 channels, which cause $Ca^{2+}$ influx into the cells by $H_2O_2$ binding (Miller, 2006).

In Japan, green teas, some of which are catechin-enriched, are popular. Green tea has several constituents which are good for human health, and has attracted inter-

* Corresponding author. Tel./fax: +81 83 933 5762.
*E-mail address:* aoshima@yamaguchi-u.ac.jp (H. Aoshima).

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national attention. The typical components of green tea include catechin, caffeine, dietary fibre, and vitamin C, and are expected to have physiologic activity, including antioxidative (Hanasaki et al., 1994), anti-cancerous (Fujiki et al., 1999; Lin, Liang, & Lin-Shiau, 1999), anti-mutagenic (Conney, Lu, Xie, & Huang, 1999; Shimoi, Nakamura, Tomita, & Kada, 1985), and anti-obesity (Sayama, Ozeki, Taguchi, & Oguni, 1996) effects. The anti-oxidative effects of catechin and its derivatives are particularly important, since the amounts of these compounds in green tea are very high.

A correlation between the production of $H_2O_2$ and total concentration of polyphenols in beverages has been reported (Aoshima & Ayabe, 2007). Though ascorbic acid is added to teas sold in polyethylene terephthalate (PET) bottles, $H_2O_2$ is produced significantly after the opening of their caps (Aoshima & Ayabe, 2007; Wee, Long, Whiteman, & Halliwell, 2003). Therefore, it is necessary to develop methods to prevent the production of $H_2O_2$ in beverages drunk several hours after the opening of their caps. PET bottles with a volume of 1.5 or 2 litres filled with green tea or Oolong tea have become popular in Japan recently and many Japanese, especially young people, consume them over a few days. We have reported that the addition of catalase, or compounds which have reductive activity or lower the pH of the beverage, reduced the amount of $H_2O_2$ produced in catechin-enriched green tea. However, because of problems such as a lack of stability or change in taste, the development of new simple and safe methods is desired.

In this paper, we examined the influence of aqueous extracts from the peel of various citrus fruits on $H_2O_2$ production in catechin-enriched green tea, and found that water-soluble compounds with a range of molecular weights had a very strong preventive effect on its production.

## 2. Materials and methods

### 2.1. Chemicals and samples

Xylenol orange and butylated hydroxytoluene (BHT) were purchased from Wako Pure Chemical Industry, Ltd., Osaka, Japan. Ammonium ferrous sulfate was purchased from Nacalai Tesque, Kyoto, Japan. TOYOPEARL HW-55F for gel chromatography was obtained from Tosoh Corporation, Tokyo, Japan. All chemicals used were of reagent quality. Apple (*Malus pumila* P. Mill), yuzu (*Citrus junos* Tanaka), orange (*Citrus sinensis* Osbeck), grapefruit (*Citrus paradisi* Mackfady.), natsumikan (*Citrus natsudaidai* Hayata), lemon (*Citrus limon* Burm.f), lime (*Citrus aurantifolia* Christm.), and green tea packed in a PET bottle, were purchased from a local market in Yamaguchi, Japan. The green tea contained 1.57 g/l of catechin derivatives, 232 mg/l of caffeine and an undefined amount of added L-ascorbic acid.

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

The concentration of $H_2O_2$ was measured (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 concentration of $H_2O_2$ was measured, were added to the FOX reagent (3 ml) and vortexed for 5 s. After 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 using a molar extinction coefficient of 43 $M^{-1}$ $cm^{-1}$ at an absorbance wavelength of 240 nm.

### 2.3. Preparation of fruit juice and citrus peel aqueous extract

The fruit juice was prepared by crushing the fruits in a mortar and filtrating them through a cheese-cloth. The peel of the citrus fruit was removed without the pith, using a knife, and minced into small pieces. The minced citrus fruit peel (5 g) was ground down in a mortar, and deionised water (5 ml) was added. After centrifugation at 12,000 rpm for 15 min at room temperature, the supernatant was used for experiments.

### 2.4. Reduction of $H_2O_2$ levels in catechin-enriched green tea by citrus peel extract

Fifty microlitres of each citrus fruit peel extract or deionised water were added to 450 μl of catechin-enriched green tea. To this mixture, 500 μl of 100 mM phosphate buffer (pH 7.4) or deionised water was added. The mixture was vortexed for 5 s and then incubated at 60 °C for 24 h in the dark. The amount of $H_2O_2$ in the mixture was then measured using the Fox assay.

When the effect of the concentration of extract on the production of $H_2O_2$ was examined, the extract of grapefruit peel was diluted with deionised water. One volume of the diluted extract was added to 9 volumes of catechin-enriched green tea. The amount of $H_2O_2$ in the mixture was measured after incubation at 60 °C for 24 h.

When the time dependence of the production of $H_2O_2$ was examined, a mixture of one volume of the grapefruit peel aqueous extract and 9 volumes of catechin-enriched green tea was incubated at 60 °C for various periods and the amount of $H_2O_2$ was measured.

### 2.5. Degradation of $H_2O_2$ by the grapefruit peel aqueous extract

The grapefruit peel aqueous extract was incubated at 60 °C for 24 h in the dark, in order to inactivate enzymes.

One volume of grapefruit peel extract or deionized water was added to 9 volumes of 1 mM $H_2O_2$ solution. A mixture of deionized water and 1 mM $H_2O_2$ solution was used as a control. The mixture was vortexed for 5 s and then incubated at 25 °C for various periods in the dark. Then, the amount of $H_2O_2$ was measured with the Fox assay.

### 2.6. Separation of the grapefruit peel extract by extraction with different organic solvents

Five millilitres of the aqueous extract of grapefruit peel were mixed with 5 ml of chloroform. The mixture was vortexed for 10 s and then centrifuged at 2000 rpm for 20 min at room temperature. The chloroform and aqueous layers were separated by a separating funnel. The chloroform in the chloroform fraction was evaporated with an evaporator and the residue was dissolved in 5 ml of ethanol. We describe this solution as the chloroform fraction of the grapefruit peel extract. Similar procedures were carried out for the aqueous fraction by adding ethyl acetate and butanol. Since, it was difficult to evaporate the butanol, we used the butanol layer as a butanol fraction without replacing it with ethanol. Thus, the grapefruit peel extract was separated into four fractions; chloroform, ethyl acetate, butanol, and aqueous fractions.

One volume of each of the four fractions was added to 9 volumes of catechin-enriched green tea. As a control, one volume of ethanol, butanol, or deionised water was added to 9 volumes of the green tea. The mixtures were vortexed for 5 s and then incubated at 60 °C for 24 h in the dark. Then, the amount of $H_2O_2$ was measured using the Fox assay.

### 2.7. Separation of grapefruit peel aqueous extract by gel-chromatography

Five millilitres of the aqueous extract of grapefruit peel were separated through a column (2.0 × 16.5 cm) of TOYOPEARL HW-55F, using deionised water as an eluent. The eluent flowed at 0.9 ml/min, was analysed by measuring ultraviolet absorption at 254 nm, and was fractionized to 3.0 ml/tube. Every fraction was incubated with the tea at 60 °C for 24 h in the dark, and its activity in reducing production of $H_2O_2$ was measured with the FOX assay.

## 3. Results

Apple, lemon, yuzu, and grapefruit juice, and the aqueous extract of grapefruit peel, were added to the catechin-enriched green tea, and the aqueous extract of grapefruit peel, and the mixtures were incubated at 60 °C for 24 h. $H_2O_2$ concentrations were measured with the FOX assay (Table 1). The pH of the green tea was about 4.5, and addition of the juice to the green tea decreased the pH to about 3.0. Without adjustments of pH, all samples examined reduced $H_2O_2$ production in the green tea. The aqueous extract of grapefruit peel reduced the production most effectively. The effect of grapefruit juice and the grapefruit peel extract on the production of $H_2O_2$ in the green tea was measured at pH 7.4. The aqueous extract reduced the level of production, but the juice lost all reducing activity at pH 7.4. The similar results were obtained for yuzu, Japanese citrus fruit.

Table 1
The effects of fruit juices and the aqueous extract of grapefruit peel on $H_2O_2$ production in catechin-enriched green tea

| | $H_2O_2$ concentration (mM) | |
|---|---|---|
| | No pH adjustment | pH 7.4 |
| Apple juice | 0.317 ± 0.022 | Not measured |
| Lemon juice | 0.052 ± 0.001 | Not measured |
| Grapefruit juice | 0.144 ± 0.005 | 1.432 ± 0.020 |
| Grapefruit peel | 0.001 ± 0.000 | 0.068 ± 0.004 |
| Control | 0.665 ± 0.022 | 1.411 ± 0.021 |

We examined the effects of aqueous extracts of the peel of various citruses. All extracts of the citruses examined, orange, grapefruit, and natsumikan, lemon, yuzu, and lime, reduced the amount of $H_2O_2$ produced in catechin-enriched green tea, when incubated with the tea at pH 7.4 and 60 °C for 24 h (Fig. 1). The effect of the extracts on the $H_2O_2$ production in the green tea was examined at 60 °C both to remove activity of catalase in the extracts and to examine their effects under severe conditions. For further experiments, grapefruit was used as a sample, since it can be purchased in local markets all the year round.

We investigated the dose-dependence of the effect of the aqueous extract of grapefruit peel on the production of $H_2O_2$ in catechin-enriched green tea (Fig. 2a). The addition of 2% (v/v) extract to the tea reduced the amount of $H_2O_2$ to 10% of the control level and the addition of 5% (v/v) extract removed the $H_2O_2$ almost completely. The reduction in the amount of $H_2O_2$ in the green tea by the grapefruit peel extract was examined at various points in the incubation and found to continue for at least 48 h (Fig. 2b).

To examine whether the extract of grapefruit peel decomposes $H_2O_2$ in green tea, the extract after incubation at 60 °C for 24 h was added to a 1 mM $H_2O_2$ solution and

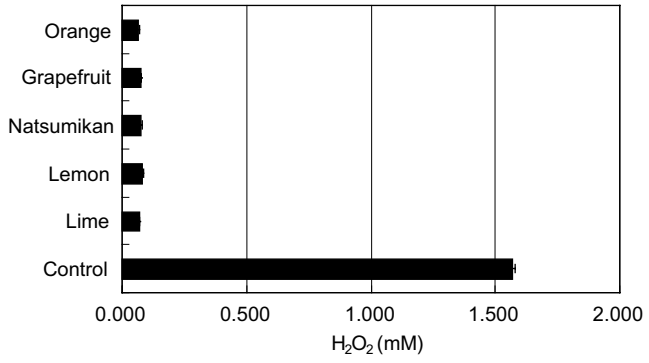


Fig. 1. The effects of aqueous extract of citrus peel on $H_2O_2$ production in green tea. The concentrations of $H_2O_2$ in mixtures were measured with the FOX assay after incubation in a plastic tube for 24 h at 60 °C and pH 7.4. Data are means ±SD, $n = 3$.

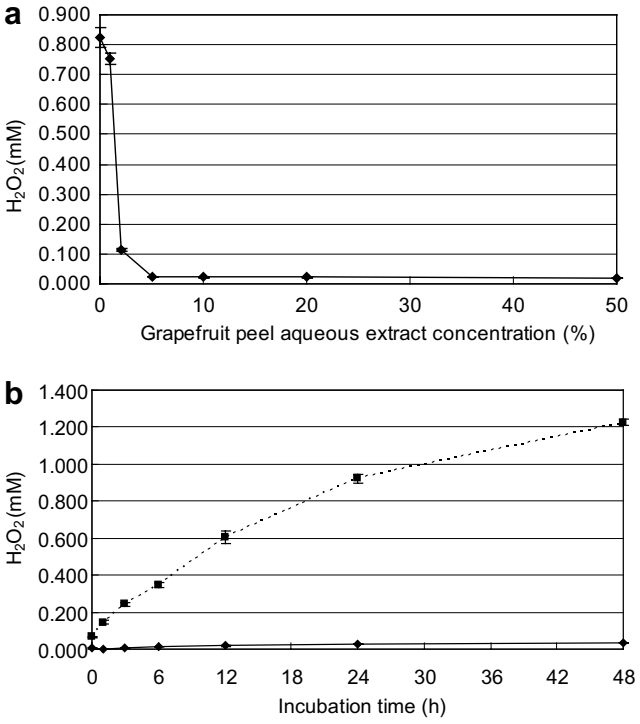


Fig. 2. (a) Dose-dependence of grapefruit peel extract on the reduction of $H_2O_2$ in green tea. Mixtures were incubated for 24 h at 60 °C. (b) Time-dependence of the aqueous extract of grapefruit peel on the reduction of $H_2O_2$ in green tea. One volume of the grapefruit peel extract (◆) or deionized water (■) was mixed with 9 volumes of green tea. Data are means ±SD, $n = 3$.

the concentrations of $H_2O_2$ in the mixture were measured at various points in time at 25 °C with the FOX assay (Fig. 3). The concentration decreased gradually with time. So it is likely that the extract decomposed the $H_2O_2$ produced in the green tea.

To characterize its active components, the aqueous extract of grapefruit peel was further separated by extraction with chloroform, ethyl acetate, and finally butanol. Since, the active constituents of the extract were present in the aqueous fraction (Fig. 4), the extract was separated by gel chromatography using TOYOPEARL HW-55F. The chromatogram was observed at 254 nm and eighty fractions were obtained by gel chromatography (data not shown). The activity reducing $H_2O_2$ in the green tea was found mainly in the initial fractions, indicating that the active compounds had a large molecular weight.

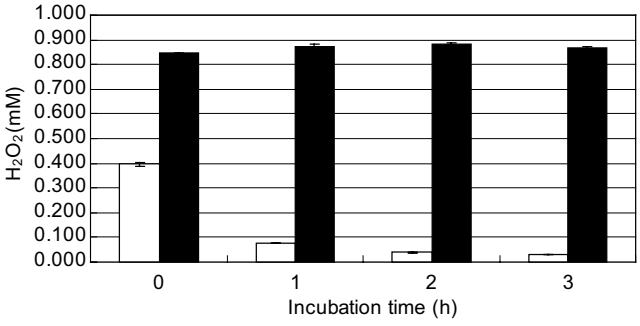


Fig. 3. Degradation of $H_2O_2$ by grapefruit peel extract incubated at 60 °C for 24 h. One volume of the extract (□) or deionised water (■) was added to 9 volumes of the green tea. Data are means ±SD, $n = 3$.

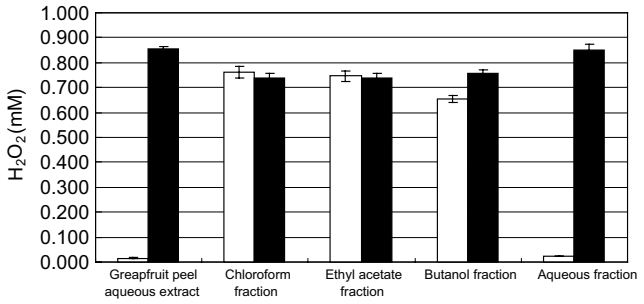


Fig. 4. The $H_2O_2$-reducing activity of grapefruit peel aqueous extract and its four fractions (□) relative to deionised water (■). Data are means ±SD, $n = 3$.

## 4. Discussion

$H_2O_2$ around 200 μM causes apoptosis in cultured rat cortical neurons (Whittermore et al., 1994) and PC12 cells (Satoh et al., 1996; Aoshima et al., 2004), through oxidative stress. Decrease of mitochondrial membrane potential and apoptosis in PC12 cells were observed in the presence of 200 μM $H_2O_2$ by flow cytometric analyses (Satoh et al., 1996a). Green tea or polyphenols in green tea caused cell death in PC12 cells or bacteria (Chai et al., 2003; Arakawa et al., 2004). Japanese government permits the use of $H_2O_2$ as a bleaching agent for foods, but demands its complete removal from the foods.

In this study, we examined the effect of fruits on the production of $H_2O_2$ in catechin-enriched green tea, since fruits are popular and safe foods, and will be accepted without hesitation by consumers. The addition of juice made from apples and citrus fruits such as grapefruit, yuzu or lemon reduced the amount of $H_2O_2$ in the green tea, but not when the mixture was adjusted to pH 7.4 by the addition of phosphate buffer, suggesting that the juices contain acidic components, such as citric acid, which lower the pH of the mixture. On the other hand, the aqueous extract of the peel reduced the level of $H_2O_2$ in the green tea even at pH 7.4 and 60 °C, suggesting that grapefruit peel contains anti-$H_2O_2$ components that are neither catalases nor acidic compounds. Since, the aqueous extracts of oranges, grapefruits, natsumikans, lemons, yuzu, and limes similarly reduced the amount of $H_2O_2$ in the green tea, citruses possibly contain common compounds which reduce $H_2O_2$ production.

The active compounds are not catalases, because boiling the extract for 10 min or addition of $NaN_3$ to the reaction mixture did not affect the activity. However, the extract lost its activity after incubation overnight in 0.12 M HCl at 60 °C.

Since, ferrous ion ($Fe^{2+}$) breaks down $H_2O_2$ via the Fenton reaction, we measured the concentration of $Fe^{2+}$ in the grapefruit peel extract and found that it was very low (data not shown). Furthermore, we examined the possibility that

the extract contained microorganisms which decomposed $H_2O_2$ in the green tea. The grapefruit peel extract was passed through a sterile filter (Millex-GS, 0.22 μm filter unit, Millipore) to remove microorganisms. The activity remained in the filtrate (data not shown), precluding the possibility that microorganisms in the extract caused the activity. At present, we do not know what the active components in the grapefruit peel are. We are now attempting to identify the active components, which may be useful for reducing the production of $H_2O_2$ and good for our health, if they are absorbed into the body.

## 5. Conclusion

Though beverages contain polyphenols beneficial to human health, $H_2O_2$ is produced in beverages after their exposure to air. The peel of citruses such as grapefruits contained components which reduced the amount of $H_2O_2$ in the green tea. These components are present in the aqueous phase and vary extensively in molecular weight. They are not catalases, acidic compounds, $Fe^{2+}$ or microorganisms. It is necessary to identify these components in the future.

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