Food Chemistry 112 (2009) 339–343

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Anti-hydrogen peroxide activity of fish and soy sauce

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ARTICLE INFO

Article history:
Received 1 November 2007
Received in revised form 14 March 2008
Accepted 22 May 2008

Keywords:
Antioxidant
Fish sauce
Hydrogen peroxide
Polyphenol
Soy sauce
Thermostable catalase

ABSTRACT

Shoyu is the Japanese name for soy sauce and the most popular liquid condiment (seasoning) used in Japanese cuisine as well as in cuisines of other oriental countries. Shoyu is prepared by digesting mold-cultured soybeans and wheat seeded with an aspergillus (koji in Japanese) in the presence of sodium chloride. Gyoshoyu is produced when the soybeans and wheat are replaced with fish. Both Shoyu and Gyoshoyu have high level of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity. Hydrogen peroxide is produced in green tea after exposure to air. To find a safe and economical method of preventing the production of H_2O_2 in green tea, effects of Shoyu and Gyoshoyu on H_2O_2 level in bottled green tea were examined. Both Shoyu and Gyoshoyu suppressed the production of H_2O_2. Gyoshoyu decomposed H_2O_2 possibly because of the presence of a thermostable catalase, while Shoyu did not. Some components of Shoyu and Gyoshoyu may be useful to suppress the production of H_2O_2 in green 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; Hara, 2001; Lambert & Yang, 2003). Not only green and black tea but also various kinds of herbal teas are very popular in Japan because of their fragrance and antioxidative activity (Matsingou, Kapsokefalou, & Salifoglou, 2001). These beverages are thought to be beneficial to both physical and mental health. 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 recently (Arakawa, Maeda, Okubo, & Shimamura, 2004; Cao, Sofic, & Prior, 1997; Elbling et al., 2005; Kajiya, Ichiba, Kuwabara, Kumazawa, & Nakayama, 2001; Long, Lan, Hsuan, & Halliwell, 1999; Nakayama, Ichiba, Kuwabara, Kajiya, & Kumazawa, 2002). H_2O_2 was produced from polyphenol-rich beverages under quasi-physiological conditions and increased in amount 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 in bottles are sold in vending machines in Japan. These beverages contain a negligible amount of H_2O_2 just after being opened, but their H_2O_2 levels gradually increase with time. Aoshima and Ayabe (2007) have reported methods to prevent the production of H_2O_2 by adding catalase or compounds which have reductive activity or lower the pH of the beverages. The addition of some herbal teas or citrus peel extracts also prevents the production of H_2O_2 in green tea (Aoshima, Hirata, & Ayabe, 2007; Ayabe & Aoshima, 2007).

Shoyu is the Japanese name for soy sauce and the most popular liquid condiment used in Japanese cuisine as well as in cuisine of other oriental countries. Recently, Shoyu has become popular not only in USA, but also in European countries. Shoyu is prepared by digesting mold-cultured soybeans and wheat seeded with an aspergillus (koji) in the presence of sodium chloride (Kataoka, 2005). Gyoshoyu is prepared when the soybeans and wheat are replaced with fish (Fig. 1).

In this paper, the total polyphenol concentration (Vinson, Proch, & Bose, 2001), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity (Blois, 1958) and amount of H_2O_2 (Long et al., 1999) in Shoyu and Gyoshoyu were measured to examine the antioxidative activity and toxicity of the sauces. Then, we examined their anti-H_2O_2 activity by adding them to a catechin-enriched green tea which produces H_2O_2. Further, we examined whether they decompose H_2O_2 or not.

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doi:10.1016/j.foodchem.2008.05.069

a) Preparation of Shoyu (soy sauce)
Steamed wheat seeded with an aspergillus (koji)
↓
Addition of boiled soybeans and sodium chloride
↓
Fermentation with lactobacilli and yeasts, and aging
↓
Separation to liquid and solid phases
↓
Pasteurization of liquid phase
↓
Packing into bottles

b) Preparation of Gyoshoyu (fish sauce)
Mixture of dried bonito and its aqueous extract seeded with an aspergillus
↓
Addition of boiled fish (bonito, red sea-bream, or shrimp) and sodium chloride
↓
Disassimilation, fermentation and aging
↓
Separation to liquid and solid phases
↓
Pasteurization of liquid phase
↓
Packing into bottles

Fig. 1. Schemes for the preparation of Shoyu (soy sauce) and Gyoshoyu (fish sauce).

2. Materials and methods

2.1. Chemicals and samples

Xylenol orange, butylated hydroxytoluene (BHT), methanol, and DPPH were purchased from Wako Pure Chemical Industry, Ltd., Osaka, Japan. Gallic acid was purchased from Nacalai Tesque, Kyoto, Japan. Folin and Ciocalteu's phenol reagent was obtained from Katayama Chemical Industry, Osaka, Japan. All chemicals used were of guaranteed reagent quality. Three kinds of Gyoshoyu (S1–S3) were supplied from Yamaki Co., Ltd., Iyo, Ehime, Japan. Three fish, bonito (S1), red sea-bream (S2), and shrimp (S3), were used instead of soybeans. Shoyu (S4; Nibishi Shoyu Co., Ltd., Koga, Fukuoka, Japan) was purchased from a local market in Yamaguchi. Catechin-enriched green tea in a polyethylene terephthalate (PET) bottle was purchased from a local market in Yamaguchi, Japan. This green tea contained 1.57 g/l catechin derivatives, 232 mg/l caffeine and an undefined amount of L-ascorbic acid.

2.2. Determination of the total polyphenol concentration with the Folin assay

The total polyphenol concentrations of Shoyu and Gyoshoyu were analyzed using the Folin assay (Aoshima & Ayabe, 2007; Vinson et al., 2001). Gallic acid was used as the standard and the polyphenol concentrations in beverages were expressed as mM gallic acid equivalent. One milliliter of sauce diluted 50 fold with deionized water was mixed with 1 ml of Folin-Ciocalteu's reagent. After vortexing for 5 s, 1 ml of a 10% (w/w) aqueous sodium carbonate solution was added to the mixture. The mixture was incubated at room temperature for 1 h and absorbance was read at 700 nm by a spectrophotometer (Hitachi U-2000A).

2.3. 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 sauce diluted two hundred fold with 50% (v/v) aqueous ethanol solution, was shaken vigorously for a short time to mix the mixture (Aoshima, Tsunoue, Koda, & Kiso, 2004; Blois, 1958). As a control, the reaction mixture was prepared similarly but the sauce was replaced with deionized water. After incubation at room temperature for 30 min, the amount of DPPH remaining was determined by measuring the absorbance at 517 nm, and the radical-scavenging activity of each sample was expressed using the ratio of the decrease in absorption of DPPH (%) relative to the control DPPH solution (100%) in the absence of the sample. That is, 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, respectively.

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

The concentration of H_2O_2 was measured as previously described (Akagawa et al., 2003; Long et al., 1999). FOX reagent was prepared by adding nine volumes of reagent 1 to one volume 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 sauces (100 μl), in which the H_2O_2 concentrations were to be 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 spectrophotometer (Hitachi U-2000 A). 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 240 nm.

2.5. Prevention of H_2O_2 production in catechin-enriched green tea by the sauces

To examine the effects of the sauces on the production of H_2O_2 in catechin-enriched green tea, 0.1 ml of sauce was added to 0.9 ml of green tea and each mixture was incubated for 24 h at 60 °C. Then the H_2O_2 concentrations in the mixtures were measured by the FOX assay.

To examine the effect of the concentration of sauce on the production of H_2O_2 in green tea, mixtures with sauce diluted by deionized water were incubated for 24 h at 25 °C. Then the H_2O_2 concentrations were measured by the FOX assay.

2.6. Decomposition of H_2O_2 by the sauces

One volume of sauce was mixed with nine volumes of 1 mM H_2O_2 and incubated for 1 h at 25 °C. The control was obtained by mixing one volume of deionized water with nine volumes of 1 mM H_2O_2. The H_2O_2 concentrations in the mixtures were measured by the FOX assay. To examine the effect of the concentration of sauce on the decomposition of H_2O_2, each sauce was diluted with deionized water. To examine the effect of preheating the Gyoshoyu (S2) on the decomposition, the sauces were heated for various periods and at various temperatures in a heating block (Taitec OTU-1B). To remove microorganisms, the sauces were passed through a sterile filter (Millex-GS, 0.22 μm filter unit, Millipore).

To examine the effect of sodium azide, an inhibitor of catalases (sodium azide), on the decomposition of H_2O_2 by the Gyoshoyu, so-

dium azide was added at various concentrations to the mixtures and the activity to break down H_2O_2 was measured.

3. Results

To examine the relationship between the total polyphenol concentration and antioxidative activity in Shoyu (soy sauce) and Gyoshoyu (fish sauce; S1–S3), we measured the polyphenol content and DPPH radical-scavenging activity of the sauces (Table 1). The total concentration of polyphenol and the DPPH radical-scavenging activity were greater for the Shoyu than Gyoshoyus.

Reportedly, H_2O_2 is produced from polyphenol-rich beverages under quasi-physiological conditions and increases in concentration with time (Akagawa et al., 2003; Chai et al., 2003). So we examined the production of H_2O_2 in Shoyu and Gyoshoyu. Only a small amount of H_2O_2 was detected (data not shown).

Since H_2O_2 is produced in polyphenol-rich beverages once the bottle has been opened, it is desirable to find easy and safe methods to prevent H_2O_2 production in beverages. Here, we examined H_2O_2 production in catechin-enriched green tea sold in a PET bottle, adding various types of Shoyu and Gyoshoyu (10%, v/v) to see whether they reduce the level of H_2O_2 at 25 °C. Fig. 2 shows that the Shoyu (S4) and Gyoshoyu (S1–S3) reduced H_2O_2 production, significantly. We next examined the dose-dependence of the suppressive effect. Gyoshoyu (S2) prevented the production of H_2O_2 even at a concentration of 0.1% (v/v), while 1% (v/v) Shoyu (S4) prevented production only slightly (about 30%).

To examine whether the Gyoshoyu (S1–S3) and Shoyu decompose H_2O_2, a mixture of each sauce with H_2O_2 was incubated for 1 h at 25 °C and the concentrations of H_2O_2 was measured by FOX assay. The Gyoshoyu (S1–S3) decomposed H_2O_2, but the Shoyu (S4) did not (data not shown). Then we examined the red sea-bream sauce with an aspergillus (S2) as a model of Gyoshoyu in comparison with Shoyu (S4) (Fig. 3).

To clarify why the Gyoshoyu (S2) breaks down H_2O_2, we conducted the following experiments. Since 4-hydroxy-5-methyl-3(2H)-franone is one of the main antioxidants in Shoyu (Kataoka, 2005), the effect on H_2O_2 was measured. However, no reduction in H_2O_2 was observed (data not shown). As ferrous ions (Fe^{2+}) are broken down via the Fenton reaction (Hauptmann & Cadenas, 1997), their concentrations in the Gyoshoyu were measured spectrophotometrically by using 1,10-phenanthroline and found to be very low (4 μM). Furthermore, the possibility was examined that Gyoshoyu contained microorganisms which decomposed H_2O_2. The Gyoshoyu was passed through a sterile filter (Millex-GS, 0.22 μm filter unit, Millipore) to remove microorganisms. The activity to break down H_2O_2 remained in the filtrate (data not shown), ruling out the possibility that microorganisms in the Gyoshoyu caused the activity.

The effect of temperature on the activity to break down H_2O_2 in Gyoshoyu was examined, since it was found that a bovine liver catalase lost its activity at 60 °C previously (Aoshima & Ayabe, 2007).

Table 1
Total polyphenol concentration and DPPH radical-scavenging activity of fish and soy sauces

Sample	Total polyphenol (mM)	Scavenging activity (%)
S1 (Bonito sauce)	13.1 ± 0.3	32.3 ± 3.7
S2 (Red sea-bream sauce)	14.5 ± 0.2	31.1 ± 2.4
S3 (Shrimp sauce)	15.5 ± 0.5	30.8 ± 1.8
S4 (Soy sauce)	30.3 ± 0.2	87.7 ± 0.1

The total concentration of polyphenols was expressed as mM gallic acid equivalents. Data are means ± SD, $n = 3$. $P < 0.05$ between the control and the sample, with Student's t-test.

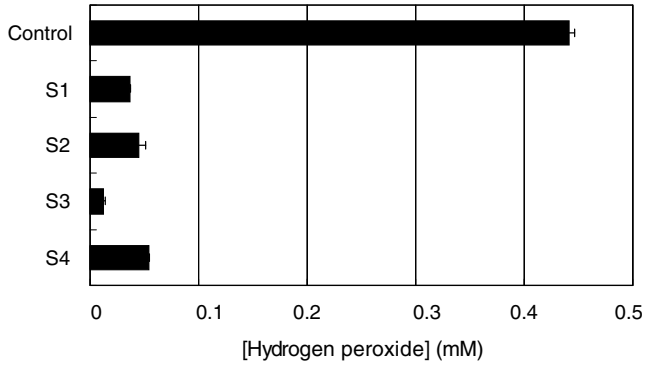

Fig. 2. Prevention of H_2O_2 production in catechin-enriched green tea by Gyoshoyu and Shoyu. A mixture of nine volumes of green tea and one volume of sauce was incubated for 24 h at 25 °C and the concentration of H_2O_2 in the mixture was measured by the FOX assay. S1: Bonito sauce, S2: red sea-bream sauce, S3: shrimp sauce, S4: soy sauce. Data are means ± SD (bars), $n = 3$. $P < 0.01$ by Student's t test for the presence versus the absence of the sauce.

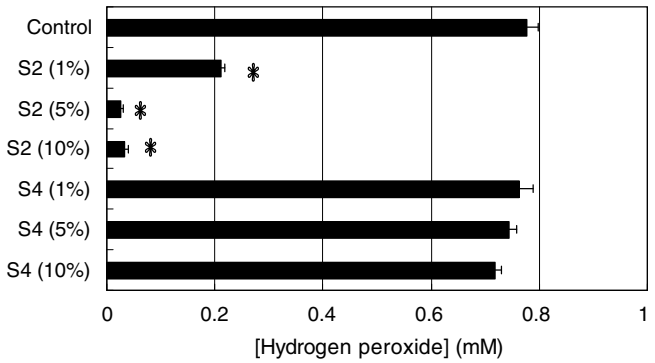

Fig. 3. Decomposition of H_2O_2 by S2 (red sea-bream sauce) and S4 (soy sauce). Mixtures of nine volumes of 1 mM H_2O_2 and the sauces at various concentrations were incubated for 1 h at 25 °C. Then the H_2O_2 concentrations in the mixtures were measured by FOX assay. Data are means ± SD (bars), $n = 3$. $P^* < 0.01$ by Student's t test between the control value and the values obtained in the presence of the sauces.

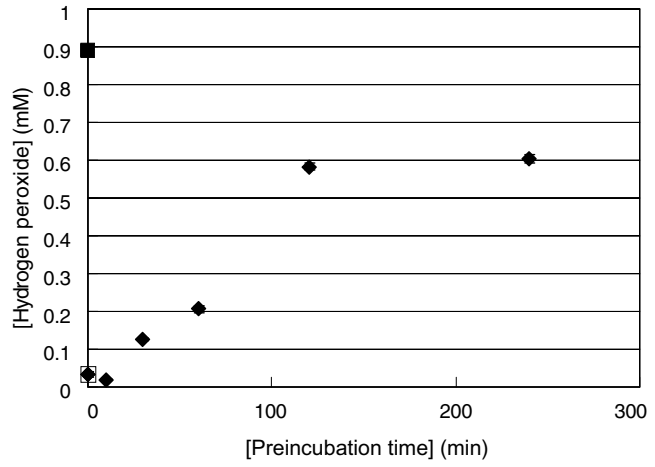

Fig. 4. Effect of preheating time at 80 °C on the decomposition of H_2O_2 by red sea-bream sauce (S2). Mixtures of nine volumes of 1 mM H_2O_2 and S2 preheated for various periods at 80 °C were incubated for 1 h at 25 °C. Then the H_2O_2 concentrations in the mixtures were measured by FOX assay. The control (■) was prepared by replacing S2 with deionized water. Data are means ± SD (bars), $n = 3$. $P < 0.01$ by Student's t test between the value at 0 min and the values obtained after preheating for more than 30 min.

Incubation at 60 or 70 °C for 1 h had no effect. However, incubation at 80 °C reduced the level of activity gradually (Fig. 4) with a complete loss after the Gyoshoyu was boiled. To confirm that the H_2O_2 was decomposed by a thermostable catalase in Gyoshoyu, sodium azide, an inhibitor of catalases, was added to the mixture of Gyoshoyu and H_2O_2. The sodium azide inhibited the decomposition of H_2O_2 by the Gyoshoyu (0.5 mM sodium azide; 26.6% inhibition).

4. Discussion

Polyphenols in beverages are popular because of their beneficial physiological effects (Bravo, 1998; Hara, 2001; Ina et al., 2002). Catechin and chlorogenic acid derivatives are polyphenols recognized as antioxidants in teas and coffee. However, the production of hydrogen peroxide (H_2O_2) from polyphenols in beverages has been reported and a mechanism for it was proposed recently (Akagawa et al., 2003; Chai et al., 2003). The toxicity of H_2O_2 has been measured *in vitro*. H_2O_2 causes apoptosis through oxidative stress (Aoshima et al., 1999; Aoshima et al., 2004; Fuchs et al., 1997; Whittermore et al., 1994). Green tea and polyphenols in green tea also caused cell death in PC12 cells and bacteria (Arakawa et al., 2004; Chai et al., 2003).

H_2O_2 was produced in green tea stored in bottles after removal of the cap and exposure to air (Aoshima & Ayabe, 2007). The addition of a catalase, a reducing agent, such as L-cysteine, or an acidic compound, such as L-aspartic acid or citric acid, had a suppressive effect. The addition of hibiscus and thorn apple teas also reduced the production of H_2O_2 in green tea, since they lowered the pH of the beverage (Aoshima et al., 2007). Moreover, the addition of an aqueous extract of citrus peel reduced the production of H_2O_2 in green tea (Ayabe & Aoshima, 2007). It is desirable to find safe and economical natural products which prevent H_2O_2 production at high temperature and pH 7.4. Ascorbic acid (vitamin C) is usually added to green teas sold in PET bottles. However, ascorbic acid itself reportedly produces H_2O_2 (Clement, Ramalingam, Long, & Halliwell, 2001; Wee, Long, Whiteman, & Halliwell, 2003).

In this paper, the effect of soy sauce on the production of H_2O_2 in the green tea was examined, since this widely used condiment is known to have various beneficial effects including antioxidative activity (Kataoka, 2005). Shoyu is usually produced from mold-cultured soybeans, wheat seeded with an aspergillus (koji), and sodium chloride. Gyoshoyu is produced from fish without soybeans and wheat, and is similar to the fish sauces used in Asian countries such as Thailand and Vietnam, which are known to have powerful antioxidative activity (Long, Kwee, & Halliwell, 2000). We obtained soy sauce (Shoyu) and three fish (bonito, red sea-bream and shrimp)-based sauces (Gyoshoyu) and examined their effect on the production of H_2O_2 in the green tea. Both Shoyu and Gyoshoyu prevented the H_2O_2 production in the green tea. Gyoshoyu decomposed H_2O_2, but Shoyu did not. This activity of H_2O_2 of Gyoshoyu (S2) decreased with incubation time at 80 °C and was lost completely after boiling. The activity in Gyoshoyu was inhibited by sodium azide, an inhibitor of catalases. The molecular weight of the active component was estimated to be more than 100 kDa through gel chromatography (data not shown). So the active compound in Gyoshoyu is estimated to be a thermostable catalase. Thermostable catalases have been found in various microorganisms such as *Bacillus stearothermophilus* (Loprasert, Negoro, & Okada, 1989) and the *Thermus* species YS 8-13 (Kagawa et al., 1999). So a thermostable catalase may be produced by the microorganisms in Gyoshoyu. Shoyu did not decompose H_2O_2 but prevented H_2O_2 production in the green tea. At present, we can explain neither what compounds in the Shoyu prevent the production of H_2O_2 in green tea nor how they induce the activity.

Gyoshoyu is useful for reducing level of H_2O_2 in foods, when it is added as a liquid condiment. To reduce H_2O_2 production in the green tea, a very small amount (0.1%, (v/v)) of Gyoshoyu can be added after the deionization of sodium chloride. If the gene for the thermostable catalase in Gyoshoyu can be cloned and the enzyme produced in great quantities in *Escherichia coli*, the catalase could be added to green tea in PET bottles.

5. Conclusions

Both soy sauce (Shoyu) and fish sauce (Gyoshoyu) had strong DPPH radical-scavenging activity. They prevented the production of H_2O_2 when added to green tea. Gyoshoyu decomposed H_2O_2 even at a very low concentration (less than 0.1% (v/v)) possibly because of the presence of a thermostable catalase. Gyoshoyu is useful for reducing level of H_2O_2 in foods or beverages.

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