Detection of Lipid Peroxidation Catalyzed by Chelated Iron and Measurement of Antioxidant Activity in Wine by a Chemiluminescence Analyzer

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We measured directly the reactive oxygen generated from a peroxide-free reaction system when a ferrous complex with nitrilotriacetic acid was oxidized to the ferric complex. Further, it was observed by a measurement of chemiluminescence that peroxidation of a lipid substrate added in the system is initiated by the Fe^{3+}-type of reactive oxygen generated. Antioxidant activity can be estimated by contrasting the reaction rates of lipid peroxidation between the systems with and without a putative antioxidant sample. By this method, the antioxidant activity, expressed as catechin equivalent, of red wines for linoleic acid peroxidation was shown to be higher than those of rosé and white wines (189−311, 84, and 37 μM for red, rosé, and white wines, respectively) because of a higher concentration of polyphenols such as flavanol and anthocyanin in red wines. The chemiluminescence measurement would be a promising method for evaluating the antioxidant potential because of its highly specific and sensitive detection of the hydroperoxide and for monitoring in situ peroxidation reaction.

Keywords: *Peroxidation; chelated iron; antioxidant activity; chemiluminescence; wine*

INTRODUCTION

Recently, the mortality from coronary heart diseases and cancers ranks high in many countries. These diseases are associated with the oxidative damage causing the production of free radicals and/or reactive oxygen species in cells or tissues when the antioxidant defenses are compromised (Harman, 1981; Ames, 1989; Steinberg et al., 1989). Free radical scavenging agents are provided in daily food stuffs. Such components as α-tocopherol, β-carotene, and ascorbic acid in the diet have been examined as potential antioxidants, assuming that they reduce the mortality from coronary heart diseases and cancers (Willett, 1994). In addition, recent studies have shown that wine polyphenolic compounds (Frankel et al., 1993, 1995; Kanner et al., 1994; Fuhrman et al., 1995; Sato et al., 1996; Verhagen et al., 1996; Teissedre et al., 1996; Larrauri et al., 1996; Simonetti et al., 1997; Paquay et al., 1997; Hurtado et al., 1997; Carbonneau et al., 1998) as well as tea polyphenols (Miura et al., 1995; Vinson et al., 1995; Kumamoto and Sonda, 1998) function as powerful antioxidants and act as anti-atherosclerotic agents (Muramatsu et al., 1986). One of the major monomeric polyphenols in both wine and tea is known to be catechins. Thus, it is understood that there is a causal relationship between the prevention of human disease associated with the oxidative damage and the oral intake of antioxidative substances.

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To determine the actual antioxidant capacity in dietary constituents or biological samples, hydroperoxides should be detected more selectively and sensitively by easier performance. In this point, the chemiluminescence (CL) method seems to be more suitable for the detection of hydroperoxide (Miyazawa et al., 1987; Kondo et al., 1994) and the measurement of antioxidant activity (Whitehead et al., 1992; Maxwell et al., 1994) than other numerous methods.

The measurement of lipid peroxidation and the antioxidant test by our CL method are based on the detection of CL excited by the reactive oxygen which is produced by the reaction of hydroperoxide and cytochrome *c* (Miyazawa et al., 1994). Hence, antioxidant activity can be evaluated by contrasting the reaction rates of lipid peroxidation between systems with and without a putative antioxidant sample.

The present paper describes the establishment of a control peroxidation system, which is composed of linoleic acid (LH) micelles with tetradecyltrimethylammonium bromide (TTAB) as a substrate and reactive oxygen generated by the autoxidation of the iron chelate complex of nitrilotriacetic acid (NTA) as an initiator, as well as the investigation of the suppressible effect of a test sample added to this control system, using the hydroperoxide-specific detection of the CL method. While LH peroxidation progressed in a CL analyzer equipped with a cell chamber, the formation rate of linoleic acid hydroperoxide (LOOH) derived from LH was monitored as the emission rate of CL, which was processed by a built-in computer in situ.

Lipid peroxidation catalyzed by the iron complex of NTA has been suggested to induce renal cell carcinoma in rats and mice (Okada et al., 1987). As the test sample for the measurement of antioxidant activity, we used wines which contain many phenolic compounds that are effective antioxidants.

MATERIALS AND METHODS

Reagents. Cytochrome *c* (type IV), TTAB, (+)-catechin, and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO). LH, luminol (3-aminophthaloyl hydrazine), ethyl acetate, and methanol were from Wako Pure Chemical Ind. (Osaka, Japan). NTA disodium salt was from Aldrich Chemical Co. (Milwaukee, WI). Folin–Ciocalteu reagent, sodium carbonate, and sodium borohydride were from Katayama Chemical Co. (Osaka, Japan). Reagents and chemicals used were commercially available extrapure products.

LH was dispersed to be 2.0 μmol/mL in 50 mM TTAB in 0.1 M borate buffer, pH 9.0, and then sonicated to prepare micelles. The LH used was peroxide free by treatment with $NaBH_4$ in methanol before the preparation of micelles. The CL reagent was prepared by dissolving 10 μM luminol and 2 μM cytochrome *c* in the above borate buffer.

Induction of LH Peroxidation and Antioxidant Test with a CL Analyzer. The CL measurements were carried out with a programmed system of the CL analyzer composed of a CLD-110 photon detector and a CLC-10 photon counter (Tohoku Electronic Ind. Co., Sendai, Japan) in an air atmosphere at 30 °C.

As a control reaction mixture for the induction of peroxidation, LH micelles (1.0 mL), 6 mM NTA (0.25 mL), distilled water (0.5 mL), and CL reagent (2.0 mL) were placed in a cell (50 mm in diameter and 10 mm in height) within a CLD-110 detector, and then the peroxidation of LH was induced by adding 0.6 mM $FeSO_4$ (0.25 mL) from the outside of the cell chamber using a syringe with a long needle. The solution in the cell was continuously mixed by a magnetic stir-bar during the reaction. The emissions of CL generated with the induction of LOOH were recorded to be at a level of 10^6 counts. The
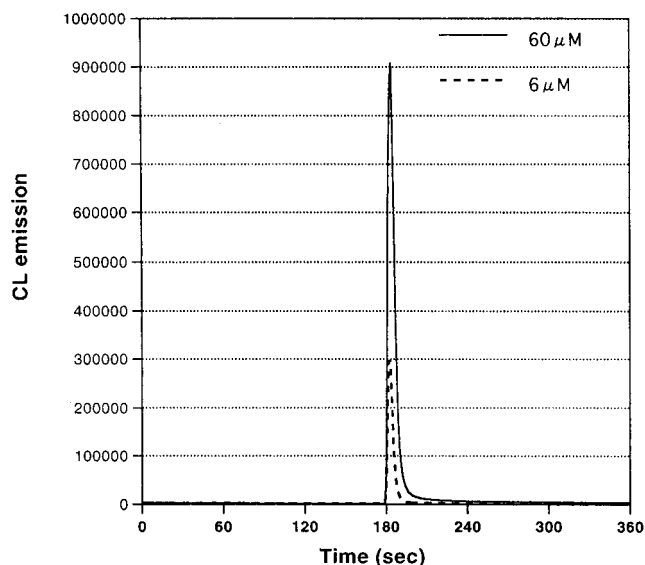

Figure 1. Chemiluminescence generated by the autoxidation of ferrous NTA to ferric NTA. For details see Materials and Methods.

measurements of the antioxidant activities of wine samples were performed using 0.5 mL of a dilute solution in place of distilled water in the above control reaction system. Dilution of wine samples was 2000–3000-fold for red wines and 500-fold for a rosé and a white wine with distilled water. The wine fractions were also diluted 1000-fold for red wines and 100–500-fold for a rosé and a white wine. The analysis of each sample was carried out in triplicate.

Wine Samples and Fractionation. Three red wines of Tokappu (a blended type), Kiyomi (made from variety Kiyomi grapes), and Silpaque (made from Zweigelt rebe and Kiyomi grapes), and a rosé and a white wine of Tokappu, were produced by Tokachi-Ikeda Research Institute for Viticulture and Enology (Hokkaido, Japan) in 1997.

The fractionation of wine was achieved using a cartridge column of Bond Elut CH (Varian, Harbar, CA) which was conditioned by the sequential wash with 10 mL each of methanol and distilled water. Four milliliters of 2-fold-diluted wine with distilled water was applied to the cartridge column. The column was washed successively with distilled water (5 mL), ethyl acetate (8 mL), and 1% HCl in methanol (8 mL) to obtain a phenolic acid fraction, flavanol fraction, and anthocyanin fraction, respectively. The water eluant was filled up to 10 mL with distilled water. The latter two fractions were evaporated to dryness, and the residues were redissolved and filled up 10 mL with distilled water.

Total Phenolic Content. The total phenolic content of wine samples was determined according to the Folin–Ciocalteu method (Singleton and Rossi, 1965) using gallic acid as a standard. The wine samples were diluted 50-fold for the red wines and 10-fold for the rosé and white wines with distilled water. One milliliter of dilute sample and 5 mL of Folin–Ciocalteu reagent were mixed in a screw-capped tube, then kept for 5 min at 50 °C. After cooling, 4 mL of 10% Na_2CO_3 solution was added. The color developed sample solutions were measured by a spectrophotometer (Ubest-V530, JASCO, Tokyo) at 765 nm.

RESULTS

Generation of Reactive Oxygen from the Iron Chelate Complex of NTA. Figure 1 shows the CL pattern emitted from the reaction reagent mixture without the LH substrate. The measurement was started after the addition of 50 mM TTAB in the buffer (1.0 mL), CL reagent (2.0 mL), 6 mM NTA (0.25 mL), and distilled water (0.5 mL) to the reaction cell in a CL
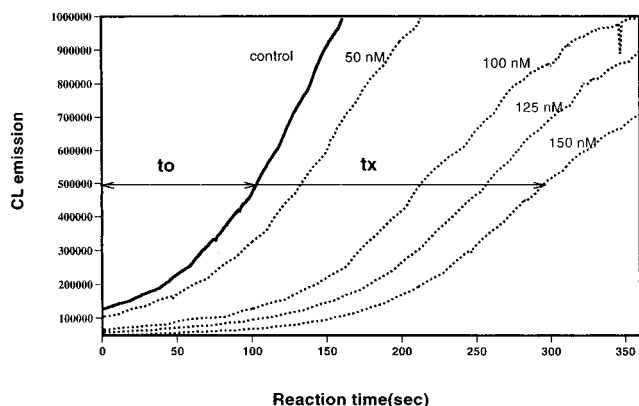
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Figure 2. Effect of the presence of catechin on a control linoleic acid peroxidation monitored by a chemiluminescence analyzer. Reaction conditions are given in the text. Solid line, a control LH peroxidation; dotted lines, those of each concentration of catechin added to the reaction mixtures; *to,* time to attain 5×10^5 counts of CL emission in the control system; *tx,* time to attain 5×10^5 counts of CL in the catechin-added system.

Figure 3. Correlation between the ratio of *tx/to* (antioxidant potentiality) and the concentration of catechin added to the linoleic acid peroxidation system.

analyzer. After 3 min, the 6 or 60 μM FeSO$_4$ (0.25 mL) was injected into the cell from the outside of the cell chamber by a syringe. Although the CL level prior to the injection of FeSO$_4$ was the same as the background, a striking emission of CL occurred simultaneously with the addition of FeSO$_4$, as observed in Figure 1. The CL emitted transitorily and returned to the basic level several tens of seconds after the emission.

Induction of LH Peroxidation and Antioxidant Test. Representative reaction patterns revealed by CL in the control peroxidation system and in that with catechin added as an antioxidant standard are shown in Figure 2. To monitor the successive reaction processes, CL was continuously measured from 1 min after the start of reaction by the addition of 0.6 mM FeSO$_4$ until reaching a level of 10^6 counts. The solid line in Figure 2 shows a control reaction curve of LH peroxidation. The control reaction curve indicated that the reactive oxygen species generated just after the addition of the ferrous reagent (FeSO$_4$) as shown in Figure 1, triggered the formation of LOOH, and the chain reaction was then propagated by the radicals produced in the reaction of LOOH and the ferrous NTA.

The four dotted lines in Figure 2 are the reaction curves where 50–150 nM catechin was added to the control system. The rates of the peroxidation in the catechin-added system were slow, compared to that of the control peroxidation system revealed by the solid line, the suppression of the peroxidation being proportional to the concentration of catechin.

Evaluation of Antioxidant Activity. Figure 3 shows a plot of *tx/to* value against the concentration of catechin added to the LH peroxidation induction system, where *to* is a time (s) to attain 5×10^5 counts of CL in the control system and *tx* is that in the catechin added system.

Antioxidant Activity of Wine. The total phenolic contents and the antioxidant activities of wines and their fractions separated by solid-phase extraction on the Bond Elut CH column are shown in Figure 4A and 4B, respectively. Measurements of antioxidant activity of each wine and its fraction were performed using the sample in place of the catechin standard in the same manner as in Figure 2.
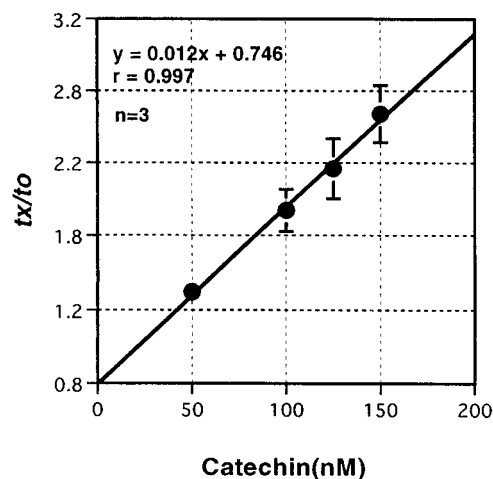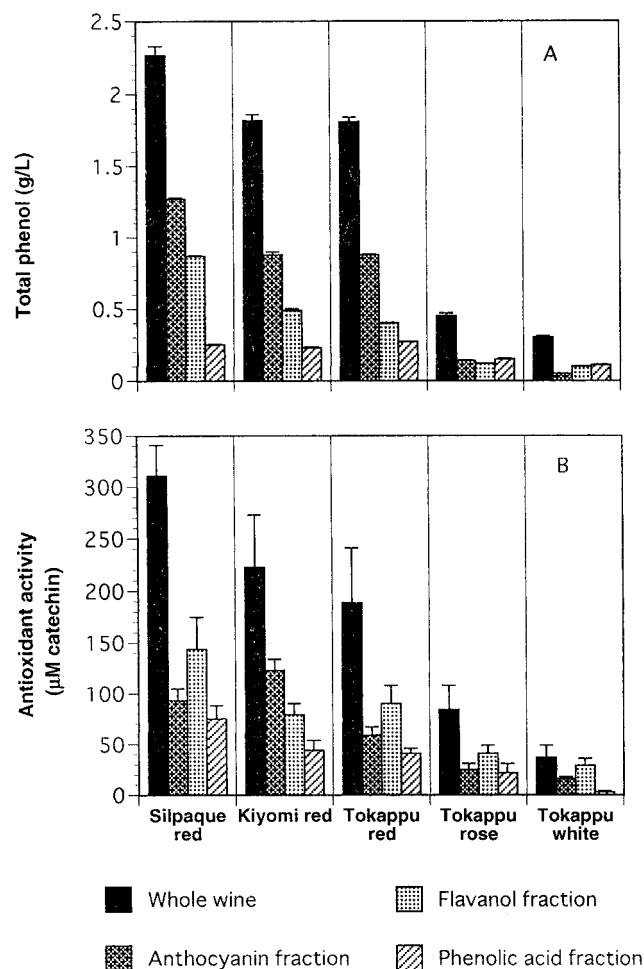
The antioxidant activity of each sample expressed as

Figure 4. Histograms of the antioxidant activity (B) and total phenol contents (A) of wines and their fractions tested.

the catechin equivalent (μM catechin) was calculated by applying *tx/to* obtained from *tx* of each sample and *to* (control) to a regression equation of the catechin standard plot shown in Figure 3. The antioxidant activities of three red wines, Silpaque, Kiyomi, and Tokappu, were 311, 223, and 189, respectively, whereas those of the rosé and white wines (both Tokappu) were 84 and 37, respectively (Figure 4B).

DISCUSSION

Okada et al. (1987) reported a high incidence of renal cell carcinoma in the ferric NTA-dosed rats. Because the iron chelate complex of NTA has a potential activity for the induction of lipid peroxidation and the DNA base oxidation (Aruoma et al., 1989; Fukuzawa et al., 1991), it was suggested that the initiation mechanism of carcinogenesis in the kidney was involved in the generation of reactive oxygen species leading to the lipid peroxidation catalyzed by the iron−NTA system (Ebina et al., 1986; Inoue and Kawanishi, 1987; Fodor and Marx, 1988; Aruoma et al., 1989; Hamazaki et al., 1989). Fukuzawa et al. (1991) showed that the induction of peroxidation of LH micelles with TTAB could be stimulated by the ferrous complex of NTA in the absence of peroxides, and they suggested that such peroxidation was caused by the generation of Fe^{3+}-type reactive oxygen due to the autoxidation of ferrous NTA to ferric NTA.

We examined by CL measurement whether the generation of reactive oxygen is simultaneous with the oxidation of the ferrous to the ferric complex of NTA. The emission of CL produced by the oxidation of luminol to the excited states is the direct evidence for the autoxidation of ferrous NTA to ferric NTA, inducing generation of Fe^{3+}-type reactive oxygen. The concentrations of $FeSO_4$ added in a system in Figure 1 were $1/10$ and $1/100$ of that used for the induction of the LH peroxidation system in Figure 2.

The present detector (CLD-110) is configured to protect the photomultiplier by regulating a chopper on the gate control circuit to close at counts over 10^6 per gate time (1 s). Therefore, the $FeSO_4$ concentration in Figure 1 was reduced to keep the generation of CL below 10^6 counts/s in order to observe the reactive oxygen.

Fukuzawa et al. (1991) found that the peroxide-independent lipid peroxidation, like the control reaction in Figure 2 (solid line), did not occur in the system using other chelators. This suggests that the redox potential of the iron complex of NTA is higher than those of other chelators such as ethylene-diaminetetraacetic acid and ethylene glycol bis(β-aminoethyl ether) N,N-tetraacetic acid.

Figures 2 and 3 show the highly dependent relation between catechin concentrations and the antioxidant activities measured by the CL method. Because the correlation coefficient between the *tx/to* value and catechin concentration was 0.997, the relative antioxidant activity against a control peroxidation can be estimated by the ratio of *tx/to* (Figure 3). Similarly, the standard antioxidant plot as shown in Figure 3 (catechin plot) can be obtained using Trolox (VE analogue) and ascorbic acid.

The major phenolics in red wine were catechins [(+)-catechin, (−)epicatechin] according to the chromatographic analysis of the flavanol fraction (data not shown). So, we expressed the results of the antioxidant activities as catechin equivalent. The CL method can be applied to the highly specific and sensitive antioxidant test.

The antioxidant activity of red wines for LH peroxidation was confirmed to be higher than rosé and white wines because of a higher concentration of phenolics in red wines produced in Hokkaido prefecture (Figure 4). Similar results from the various wines and by the various methods, excluding the CL method, have been reported as cited in Introduction. The phenol contents of wines and their fractions were generally correlated to their antioxidant activities ($r = 0.936$) by a simple regression analysis. The phenolic compounds in wines were separated into three phenol types, i.e., phenolic acid, flavanol, and anthocyanin fractions, by solid phase extraction. The high level of antioxidant activity of red wines was caused by their high flavanol and anthocyanin contents. Moreover, the anthocyanin fraction of Silpaque and Tokappu (red wine) showed lower antioxidant activity than the corresponding flavanol fractions, although the total phenolic contents were usually higher in the former fractions than in the latter. Thus, the anthocyanin fractions of Silpaque and Tokappu (red wine) indicated less correlation between the phenolic content and the antioxidant activity than that of Kiyomi among red wines tested. The compositional and structural differences of anthocyanin classes in the various red wines may influence their antioxidant activities.

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