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Evaluation of antioxidant activity of vanillin by using multiple antioxidant assays

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ABSTRACT

Background: Vanillin, a compound widely used in foods, beverages, cosmetics and drugs, has been reported to exhibit multifunctional effects such as antimutagenic, antiangiogenetic, anti-colitis, anti-sickling, and antianalgesic effects. However, results of studies on the antioxidant activity of vanillin are not consistent.
Methods: We systematically evaluated the antioxidant activity of vanillin using multiple assay systems. DPPH radical-, galvinoxyl radical-, and ABTS•$^+$-scavenging assays, ORAC assay and an oxidative hemolysis inhibition assay (OxHLIA) were used for determining the antioxidant activity.
Results and conclusion: Vanillin showed stronger activity than did ascorbic acid and Trolox in the ABTS•$^+$-scavenging assay but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays. Vanillin showed much stronger antioxidant activity than did ascorbic acid and Trolox in the ORAC assay and OxHLIA. In the ABTS•$^+$-scavenging assay, ORAC assay and OxHLIA, vanillin reacted with radicals *via* a self-dimerization mechanism. The dimerization contributed to the high reaction stoichiometry against ABTS•$^+$ and AAPH-derived radicals to result in the strong effect of vanillin. Oral administration of vanillin to mice increased the vanillin concentration and the antioxidant activity in plasma. These data suggested that antioxidant activity of vanillin might be more beneficial than has been thought for daily health care.
General significance: Based on the results of the present study, we propose the addition of antioxidant capacity to the multifunctionality of vanillin.

1. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most popularly used flavoring components extracted from the seedpods of *Vanilla planifolia* and is widely used in foods, beverages, cosmetics, and drugs. Concentrations of vanillin used in food and beverage products range widely from 0.3 to 33 mM [1]. It is expected that the high level of vanillin intake from foods and beverages will have some effects on human health. Over the past two decades, the antimutagenic properties of vanillin have been studied by many researchers. In 1986, Ohta et al. first showed the antimutagenic effect of vanillin on mutagenesis induced by 4-nitroquinoline 1-oxide, furylfuramide, captan or methylglyoxal in *Escherichia coli* [2]. Subsequently, vanillin was reported to significantly reduce mutations induced by ultraviolet light or X-ray in mammalian cells both *in vitro* and *in vivo* [3–5]. Mitomycin C- and methylmethane sulphonate-induced mutations in somatic cells of *Drosophila melanogaster* and mouse bone marrow cells were also reduced by vanillin [6,7]. It has been suggested that the antimutagenic property of vanillin is achieved by recA-dependent recombinational repair enhancement, error-prone SOS repair inhibition, non-homologous DNA end-joining inhibition, and reactive oxygen species (ROS) scavenging [8].

In general, ROS generated *in vivo*, such as hydroxyl radical (HO•) and peroxyl radical (ROO•), are highly unstable and reactive. It seems crucial to quench ROS as fast as possible before they attack biomolecules and cause harm. There are many different kinds of evaluation reports for the antioxidant activity of vanillin against ROS and other radicals, though the ROS-scavenging activity as mentioned above seems to be one of the factors for the antimutagenic property of vanillin. It has been well reported that the antioxidant ability of vanillin is very low: for example, vanillin had the weakest superoxide anion scavenging activity in five tested phenolic compounds and had almost no antioxidative activity against lipid peroxidation [9], and vanillin exhibited no or little antioxidant activity in a 1,1-diphenyl-picrylhydrazyl (DPPH) radical-scavenging assay [10–12], in a β-carotene decolorization assay [11], and in linoleic acid and cholesterol oxidation assays [13]. However, it has also been reported that vanillin inhibited protein oxidation and lipid peroxidation induced by photosensitization in rat liver mitochondria [1] and that vanillin exhibited hydroxyl radical [14] and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•$^+$) [15] scavenging activities. Furthermore, it has been reported that vanillin was a poor antioxidant and that vanillin radicals promoted oxidation of glutathione, sulfhydryl groups in ovalbumin, and NADPH [16]. Therefore, there is no consistency in results of studies on the antioxidant activity of vanillin.

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We have assessed the antioxidant activities with attention to the following proposals and points of view: 1) Niki et al. reported that there are two types of antioxidant that scavenge radicals quickly and quench many radicals, and they proposed assessing reactivity based on both reaction rate and stoichiometry [17], 2) the activities of some antioxidants vary depending on the assay method, and thus the use of multiple methods is recommended [17–20], and 3) comparative studies using common antioxidants are essential to clarify the biological significance of the activities of samples. We found that 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), a stable ascorbic acid derivative, *per se* exerted radical-scavenging activity toward unnatural model radicals, such as DPPH radical [21–24] and ABTS$^{\bullet+}$ [23,25]. The chemical properties of AA-2G as a radical scavenger were greatly different from those of ascorbic acid, in that the reaction rate with these model radicals of AA-2G was much slower than that of ascorbic acid, but the long-term radical-scavenging ability per molecule of AA-2G was superior to that of ascorbic acid. We also found by using a cell-based antioxidant assay system, oxidative hemolysis inhibition assay (OxHLIA) that the radical-scavenging activity of AA-2G was biologically relevant [26]. A stereoisomer of AA-2G, 2-*O*-β-D-glucopyranosyl-L-ascorbic acid, was also found to have long-term radical-scavenging ability similar to that of AA-2G [27]. Recently, we reassessed the antioxidant activity of arbutin using five *in vitro* assay systems, though arbutin has been reported to possess weak antioxidant activity compared to that of its precursor, hydroquinone [28]. We found that arbutin exerted strong antioxidant activity comparable or even superior to that of hydroquinone. However, we have felt that *in vivo* experiments are necessary for a complete understanding of these antioxidant activities.

Hence, in this study, we systematically evaluated the antioxidant activity of vanillin using multiple assay systems. We first performed assays using model radicals, DPPH radical, galvinoxyl radical and ABTS$^{\bullet+}$. We then evaluated its antioxidant activity by ORAC assay and OxHLIA using physiologically relevant peroxyl radicals. Finally, we confirmed the antioxidant activity of vanillin by oral administration of vanillin to mice. Furthermore, a key intermediate for the antioxidant activity of vanillin was identified in the reaction with ABTS$^{\bullet+}$ and AAPH.

2. Materials and methods

2.1. Chemicals

Vanillin was obtained from Nacalai Tesque (Kyoto, Japan). Ascorbic acid, sodium fluorescein, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and heparin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Galvinoxyl free radical and vanillic acid were from Tokyo Chemical Industry (Tokyo, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), *trans*-ferulic acid, H_2O_2 (3%) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich Chemical (Milwaukee, WI). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), horseradish peroxidase (HRP; type VI-A, essentially a salt-free 1310 units/mg solid) and protocatechuic acid were from Sigma Chemical (St. Louis, MO). Sheep erythrocytes were from Nippon Bio-Supp. Center (Tokyo, Japan). Reagents were used without further purification. All water used was Milli-Q grade.

2.2. DPPH radical-scavenging assay

The DPPH radical-scavenging activities were assessed as described previously [23]. Briefly, DPPH radical (100 μM) was mixed with an antioxidant (20 μM) in 60% ethanol/40% citric acid–sodium citrate buffer (10 mM, pH 6). The reaction was carried out at room temperature for 2 h. The decrease of DPPH radical concentration was monitored by measuring the absorbance at 524 nm with a spectrophotometer (Hitachi U-1900, Tokyo, Japan).

2.3. Galvinoxyl radical-scavenging assay

The galvinoxyl radical-scavenging activities were assessed as described previously [23]. Briefly, galvinoxyl radical (100 μM) was mixed with an antioxidant (20 μM) in 60% ethanol/40% citric acid–sodium citrate buffer (10 mM, pH 6). The reaction was carried out at room temperature for 2 h. The decrease of galvinoxyl radical concentration was monitored by measuring the absorbance at 432 nm with a spectrophotometer.

2.4. ABTS$^{\bullet+}$-scavenging assay

The ABTS$^{\bullet+}$-scavenging activities were assessed as described previously [25]. Briefly, ABTS$^{\bullet+}$ (100 μM) generated with an ABTS/H_2O_2/HRP system was mixed with an antioxidant (10 or 20 μM) in citric acid–sodium citrate buffer (50 mM, pH 6) containing 2% EtOH. The reaction was carried out at room temperature for 2 h. The decrease of ABTS$^{\bullet+}$ concentration was monitored by measuring the absorbance at 730 nm with a spectrophotometer. The 50% effective concentration (EC_{50}) value was also determined as the concentration of vanillin required to give 50% of the absorbance shown by a blank test. Vanillin (20, 30 and 40 μM) was reacted with ABTS$^{\bullet+}$ (100 μM) at room temperature for 20 and 120 min.

2.5. HPLC analysis of the reaction mixture of vanillin with ABTS$^{\bullet+}$

Vanillin (50 μM) was reacted with ABTS$^{\bullet+}$ (250 μM) generated with an ABTS/H_2O_2/HRP system in citric acid-sodium citrate buffer (50 mM, pH 6) containing 5% DMSO. An aliquot of the reaction mixture was periodically withdrawn and directly subjected to HPLC analysis. The HPLC analyses were carried out with a system consisting of a Hitachi L-7100 pump, L-7420 UV–VIS detector, L-7300 column oven, and D-2500 chromato-integrator. The separation of vanillin and reaction products was achieved by isocratic elution from an Inertsil ODS-3 column (4.6 i.d.×250 mm, 5 μm, GL Sciences Inc., Tokyo, Japan) kept at 40 °C with MeOH/H_2O/acetic acid (40:59:1, v/v) at a flow rate of 0.7 ml/min. The absorbance at 275 nm was monitored.

2.6. Purification and structural determination of the reaction product of vanillin with ABTS$^{\bullet+}$

Vanillin (1.0 g, 6.57 mmol) was dissolved in 20 ml of EtOH and then mixed with 329 ml of 10 mM ABTS$^{\bullet+}$ (3.29 mmol). The solution was diluted with 0.1 M citric acid-sodium citrate buffer (pH 6) to give 1000 ml of a mixture of vanillin and ABTS$^{\bullet+}$. The solution was reacted for 30 min at 30 °C. The reaction mixture was twice extracted with 500 ml of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 overnight to give a white precipitate. After dissolution of the precipitate by heating, the organic layer was filtered and concentrated *in vacuo* to dryness. The residue was suspended with MeOH and then filtered to give 6,6′-dihydroxy-5,5′-dimethoxy-(1,1′-biphenyl)-3,3′-dicarboxaldehyde (86.0 mg) as an MeOH insoluble compound. NMR spectra were recorded on a Varian INOVA-AS600 instrument. An electron spray ionization mass spectrum was obtained on an AB SCIEX API 4000 LC/MS/MS system using direct sample injection. ^{1}H NMR (600 MHz, DMSO-d_6) δ_H: 3.90 (6H, s, O$\underline{CH_3}$), 7.39 (2H, d, J=1.8 Hz, H-4, 4′), 7.43 (2H, d, J=1.8 Hz, H-2, 2′), 9.79 (2H, s, C$\underline{H}$O). ^{13}C NMR (150 MHz, DMSO-d_6) δ_C: 56.1 (O$\underline{CH_3}$), 109.0 (C-4, 4′), 125.2 (C-1, 1′), 127.4 (C-3, 3′), 128.5 (C-2, 2′), 148.7 (C-5, 5′), 152.0 br (C-6, 6′), 191.2 (C$\underline{H}$O). ESI-MS (negative ion mode) m/z: 301 $[M-H]^-$.

2.7. ORAC assay

The ORAC assay was carried out as described previously [27]. Briefly, fluorescein (60 nM), antioxidant (3 μM), and AAPH (18.75 mM) were incubated in 200 μl of KH_2PO_4–K_2HPO_4 buffer (75 mM, pH 7.4) at 37 °C in a 96-well plate. The fluorescence (Ex: 485 nm, Em: 520 nm) was monitored every 2 min for 70 min by Varioskan Flash (Thermo Fisher Scientific, Waltham, MA).

To determine the reaction product of vanillin with AAPH, vanillin (50 μM) was reacted with AAPH (40 mM) in phosphate-buffered saline (PBS: 150 mM NaCl, 8.1 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4, pH 7.4) containing 5% DMSO. An aliquot of the reaction mixture was periodically withdrawn and directly subjected to HPLC analysis. The HPLC analyses were carried out under the above-described conditions.

2.8. Oxidative hemolysis inhibition assay (OxHLIA)

The OxHLIA was carried out as described in previous papers [27,29]. Briefly, sheep erythrocytes suspended at a concentration of 0.7% (v/v) in PBS were incubated with 40 mM of AAPH in the presence of an antioxidant (3.1, 6.3, 12.5, 25 or 50 μM) at 37 °C with shaking. The degree of hemolysis (%) was monitored every 15 min for 165 min from the concentration of hemoglobin in the centrifuged supernatant by measuring the absorbance at 524 nm.

2.9. Contents of vanillin and its metabolites and antioxidant capacity in mouse plasma

Male ICR mice, 6 weeks old, were purchased from CLEA Japan (Tokyo, Japan). Mice that had been fed *ad libitum* on a stock diet of CE-2 (CLEA Japan) and water were fasted for 16 h prior to use. All mice were used at 7 weeks of age. They were orally given 100 mg/kg of vanillin (25 mg/ml) dissolved in 20% EtOH/PBS. At the indicated times, blood samples were collected by heart puncture in a heparin-coated syringe under sodium pentobarbital anesthesia. Each blood sample was centrifuged at 10,000×*g* for 10 min at 4 °C, and the resulting plasma was subjected to HPLC analysis and plasma ORAC assay. For HPLC analysis, to 250 μl of the resulting plasma was added 250 μl of ethyl acetate. After vortexing, the resulting mixture was centrifuged at 10,000×*g* for 5 min at 4 °C, and 150 μl of the ethyl acetate phase was removed and concentrated to dryness with a stream of air. The resulting residue was dissolved with a HPLC solvent and subjected to HPLC analysis for estimating the concentrations of vanillin, vanillic acid and protocatechuic acid. For plasma ORAC assay, 70 μl of the resulting plasma was deproteinized by adding 140 μl of acetone/H_2O/acetic acid (70:29.5:0.5, v/v). The mixture was centrifuged at 10,000×*g* for 10 min at 4 °C, and the resulting supernatant was diluted with 75 mM phosphate buffer (pH 7.4) to give a final concentration of 50 times dilution for the ORAC assay. The HPLC analysis and the ORAC assay were carried out under the above-described conditions.

3. Results

3.1. DPPH radical-, galvinoxyl radical- and ABTS•$^+$-scavenging activities

DPPH radical, galvinoxyl radical and ABTS•$^+$ are relatively stable radicals. Their characteristic colors disappear when they are quenched, and so the decrease of these radicals can be easily monitored by a spectrometer [30,31]. We assessed the DPPH radical-, galvinoxyl radical- and ABTS•$^+$-scavenging activities of vanillin in buffered solutions at pH 6 and compared them with those of vanillic acid, ferulic acid, ascorbic acid and Trolox. Vanillic acid and ferulic acid were used as known antioxidants with similar structures. Ascorbic acid and Trolox were used as standard antioxidants. The chemical structures of these compounds are shown in Fig. 1. Ascorbic acid and Trolox showed nearly the same reaction profiles in the three assays (Fig. 2), i.e., 20 μM of these antioxidants rapidly quenched ca. 40 μM of DPPH radical, galvinoxyl radical and ABTS•$^+$ within 5 min. Thus, their reaction stoichiometries (number of radical molecules reduced by one molecule of antioxidant) were about 2 and this was consistent with our previous report [23]. Vanillin scavenged little or no DPPH radical and galvinoxyl radical, while vanillic acid and ferulic acid continuously quenched both radicals for the whole experimental period (Fig. 2A, B). In the ABTS•$^+$ assay, vanillin as well as vanillic acid and ferulic acid showed significant radical-scavenging activity (Fig. 2C). Interestingly, the reaction between vanillin and ABTS•$^+$ slowly and continuously proceeded for 120 min. The 50% effective concentration (EC_{50}) of vanillin against ABTS•$^+$ was also investigated at 20 min and 120 min. The EC_{50} of vanillin markedly decreased from 40.6 μM at 20 min to 19.4 μM at 120 min, because vanillin slowly and continuously reacted with ABTS•$^+$ over a period of 120 min. The result showed that 19.4 μM of vanillin quenched 50 μM of ABTS•$^+$ for up to 120 min. Thus, the reaction stoichiometry of vanillin against ABTS•$^+$ was 2.6 at 120 min. Vanillin showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays but showed stronger activity than ascorbic acid and Trolox in the ABTS•$^+$-scavenging assay.

3.2. Reaction products of vanillin with ABTS•$^+$

Vanillin slowly and continuously reacted with ABTS•$^+$ over a period of 120 min (Fig. 2C). It is noteworthy that 1 mol of vanillin scavenged 2.6 mol of ABTS•$^+$ in 120 min of reaction, although one molecule of vanillin has only one of the oxidizable phenolic hydroxyl groups at the C-4 position (Fig. 1). To investigate characteristics of the reaction, the reaction mixtures of vanillin with ABTS•$^+$ were analyzed by HPLC. The HPLC profiles of the reaction mixture between vanillin (50 μM) and ABTS•$^+$ (250 μM) in citrate buffer (pH 6) containing 5% DMSO at 1, 5, 15, and 120 min of reaction are shown in Fig. 3. At 1 min, peaks of reaction products 1 and 2, besides vanillin, were found. The peaks of vanillin and 2 decreased with time, whereas the peak of 1 slowly increased. These results suggest that vanillin scavenged ABTS•$^+$ to generate reaction product 2 at an early stage of the reaction. Subsequently, the reaction product 2 reacted with additional ABTS•$^+$ to give the reaction product 1.

Clarifying the reaction products seemed to show the reason why the radical-scavenging activity of vanillin was higher than that of ascorbic acid and Trolox at 120 min of reaction. We tried to isolate

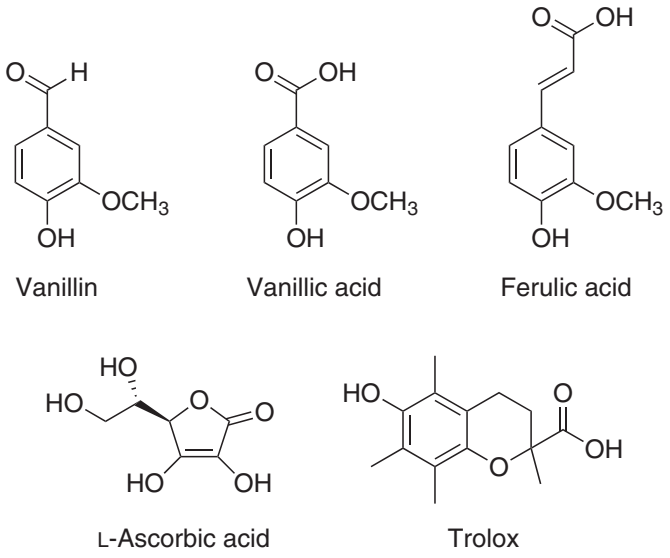


Fig. 1. Chemical structures of vanillin, vanillic acid, ferulic acid, ascorbic acid and Trolox.

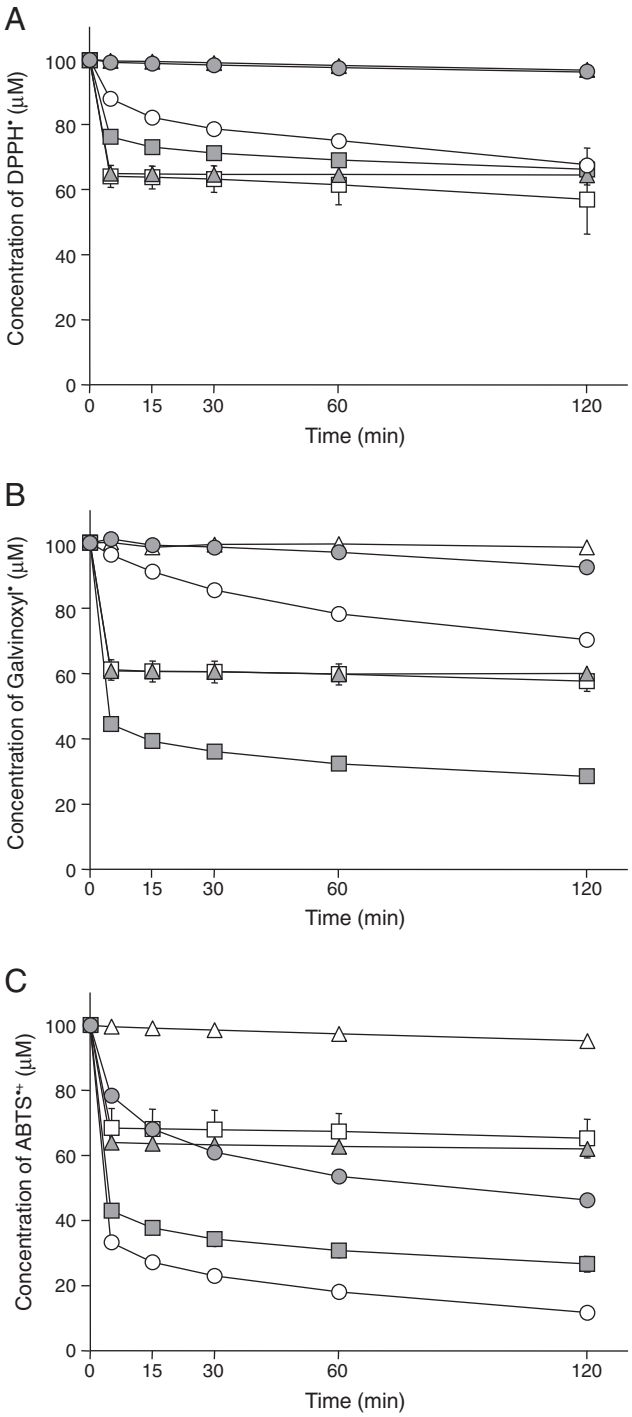


Fig. 2. Time courses of DPPH radical (A)-, galvinoxyl radical (B)- and ABTS•$^+$ (C)-scavenging reactions of vanillin, vanillic acid, ferulic acid, ascorbic acid and Trolox. Vanillin (●), vanillic acid (○), ferulic acid (■), ascorbic acid (□), Trolox (▲) (each 20 μM) or control (△) and DPPH radical or galvinoxyl radical (100 μM) were incubated at room temperature in 60% ethanol/40% citrate buffer (10 mM, pH 6). Vanillin (20 μM), vanillic acid (20 μM), ferulic acid (10 μM), ascorbic acid (20 μM), Trolox (20 μM) or control and ABTS•$^+$ (100 μM) were incubated at room temperature in 5% DMSO/citrate buffer (50 mM, pH 6). Changes in the remaining radicals were measured at the indicated times. Each value is the mean ± SD of three separate experiments. The absence of an SD bar means that the SD bar is within the symbol.

reaction products 1 and 2 from reaction mixture of vanillin (6.57 mmol) and ABTS•$^+$ (3.29 mmol) in 2% ethanol/citrate buffer (pH 6). The reaction mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 overnight to give a white precipitate. After dissolution of the precipitate by heating, the

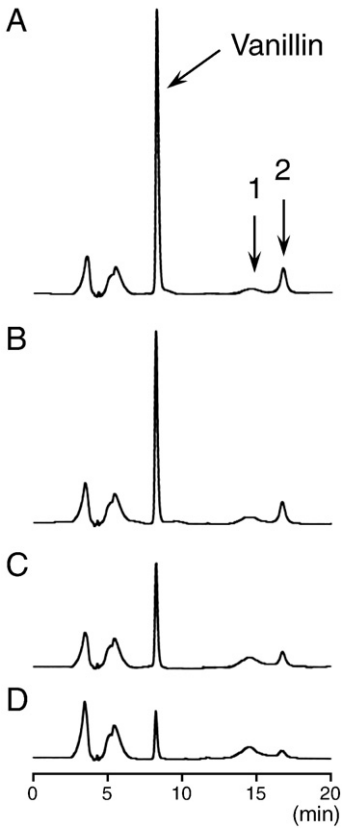
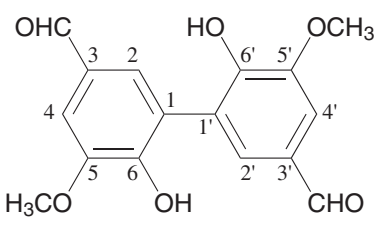


Fig. 3. HPLC chromatograms of the products of reaction of vanillin with ABTS•$^+$. Vanillin (50 μM) and ABTS•$^+$ (250 μM) were incubated in 5% DMSO/citrate buffer (50 mM, pH 6). At 1 (A), 5 (B), 15 (C), and 120 min (D), aliquots of the reaction mixture were withdrawn and analyzed by HPLC.

organic layer was filtered and concentrated *in vacuo* to dryness. The residue was suspended with MeOH and then filtered to give an MeOH insoluble compound (86.0 mg) as the reaction product 2. The reaction product 1 dissolved in MeOH was further purified by a silica gel column but was not isolated. The reaction product 2 was fully characterized and identified to be 6,6′-dihydroxy-5,5′-dimethoxy-(1,1′-biphenyl)-3,3′-dicarboxaldehyde (divanillin) by the mass spectrum and several NMR spectra (^{1}H, ^{13}C, HSQC, and HMBC) (Fig. 4).

3.3. ORAC assay

Vanillin showed stronger activity than did ascorbic acid and Trolox in the ABTS•$^+$-scavenging assay but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays. This discrepancy led us to assess the antioxidant efficacy of vanillin in more physiologically relevant assay systems, because DPPH radical, galvinoxyl radical and ABTS•$^+$ are unnatural radical species that do not exist in the human body. The ORAC assay utilizes an AAPH-derived



6,6'-dihydroxy-5,5'-dimethoxy-(1,1'-biphenyl)-3,3'-dicarboxaldehyde (divanillin)

Fig. 4. Chemical structure of one of the products of the reaction of vanillin with ABTS•$^+$.

peroxyl radical, which mimics lipid peroxyl radicals involved in lipid peroxidation chain reaction *in vivo*. Inhibition of peroxyl radical-induced oxidations of a fluorescent probe, fluorescein, by antioxidants is serially monitored [30]. Unexpectedly, vanillin had the strongest antioxidant effect in the ORAC assay. The order of inhibition was vanillin > vanillic acid ≥ ferulic acid ≫ Trolox > ascorbic acid (Fig. 5).

To investigate the characteristics of the reaction for the unexpectedly strong effect of vanillin, reaction mixtures of vanillin with AAPH-derived radicals were analyzed by HPLC. The HPLC profiles of the reaction mixture of vanillin (50 μM) and AAPH (40 mM) in PBS containing 5% DMSO at 10, 30, and 60 min of reaction are shown in Fig. 6. At 5 min, a small amount of divanillin was observed (data not shown). The peak of vanillin decreased with time, whereas the peak of divanillin gradually increased over a period of 30 min. At 60 min, the peaks of vanillin and divanillin had almost disappeared. A peak of 1 as shown in Fig. 3 was not observed in this reaction. These results suggested that the formation of divanillin resulted in the unexpectedly strong effect of vanillin.

3.4. Oxidative hemolysis inhibition assay (OxHLIA)

OxHLIA is a cell-based antioxidant assay using the same radical source as that used for the ORAC assay [26,29]. Oxidation of erythrocyte membranes by an AAPH-derived peroxyl radical induces oxidation of lipids and proteins and eventually causes hemolysis, and this hemolysis was inhibited by each antioxidant (Fig. 7A). The order of inhibition was ferulic acid (25 μM) > vanillin (25 μM) ≥ vanillic acid (25 μM) ≈ Trolox (50 μM) > ascorbic acid (50 μM), which was a little different from that observed in the ORAC assay (Fig. 5).

Concentration-dependency of vanillin for hemolysis assays is shown in Fig. 7B. The results are expressed as delayed time of hemolysis (ΔT), which was calculated by the following equation:

$$\Delta T = HT_{50}(\text{sample}) - HT_{50}(\text{control}),$$

where HT_{50} is the 50% hemolysis time. The average HT_{50} (control) was 93 ± 4 min (n = 3). The ΔT value of vanillin at 25 μM was 2.6-times greater than that at 12.5 μM. The ΔT value of vanillin at 12.5 μM was 3.2-times greater than that at 6.3 μM. The ΔT value of vanillin at 3.1 μM was scarcely observed. The inhibitory effects of vanillin on hemolysis were concentration-dependent.

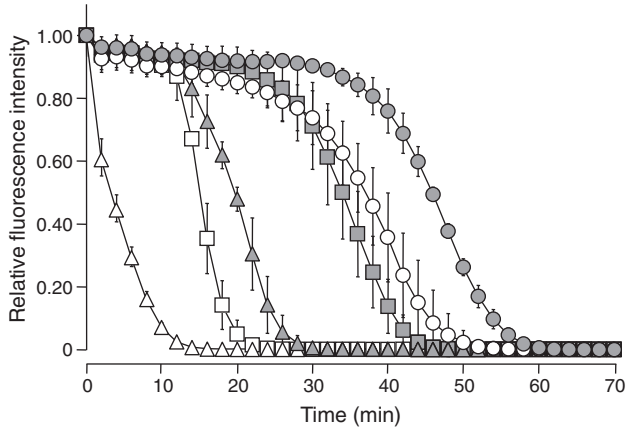


Fig. 5. ORAC assay for vanillin, vanillic acid, ferulic acid, ascorbic acid and Trolox. Reaction mixtures containing vanillin (●), vanillic acid (○), ferulic acid (■), ascorbic acid (□), Trolox (▲) (each 3.0 μM) or control (△), fluorescein (60 nM) and AAPH (18.75 mM) in 200 μl of phosphate buffer (75 mM, pH 7.4) were incubated at 37 °C for 70 min. Changes in fluorescence intensity of fluorescein were monitored. Each value is the mean ± SD of triplicate experiments. The absence of an SD bar means that the SD bar is within the symbol.

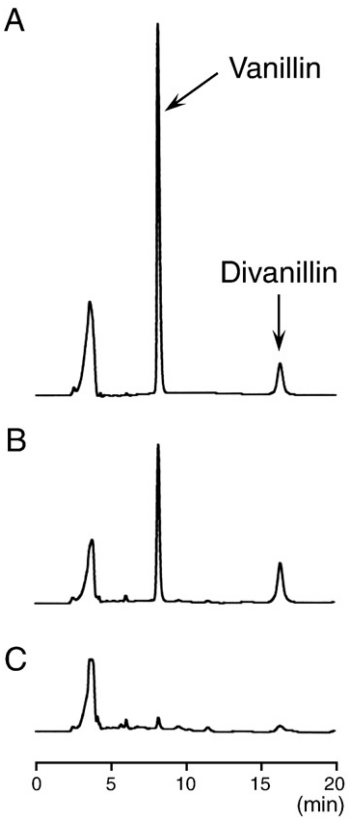


Fig. 6. HPLC chromatograms of the products of reaction of vanillin with AAPH. Vanillin (50 μM) and AAPH (40 mM) were incubated in 5% DMSO/PBS. At 10 (A), 30 (B), and 60 min (C), aliquots of the reaction mixture were withdrawn and analyzed by HPLC.

3.5. Contents of vanillin and its metabolites and antioxidant capacity in mouse plasma

In the some *in vitro* experiments, vanillin showed potent antioxidant activity compared with the activities of ascorbic acid and Trolox used as positive controls and compared with the activities of known antioxidants, vanillic acid and ferulic acid. To investigate the absorption and the antioxidant capacity of vanillin, mice were orally administrated a single dose (100 mg/kg) of vanillin. After 5, 15, 30 and 60 min, plasma samples were prepared and subjected to HPLC analysis and plasma ORAC assay. Fig. 8A shows time-course plots of the plasma concentrations of vanillin and its metabolites after oral administration of vanillin. Vanillic acid and protocatechic acid were detected as metabolites of vanillin. The concentration of vanillin sharply increased up to 5 min and then rapidly decreased until 15 min. The level of protocatechic acid reached its maximum at 5 min and then rapidly decreased until 15 min. On the other hand, the level of vanillic acid reached its maximum at 5 min and then gradually decreased with time. The maximal values of vanillin, vanillic acid and protocatechic acid at 5 min were 7.1 ± 2.1, 0.75 ± 0.09 and 1.4 ± 0.4 μg/ml, respectively. Fig. 8B shows ORAC activities in the plasma at various times after oral administration of vanillin. The plasma sample at each time showed high ORAC activity, and the order of activity was 5 min > 15 min > 30 min > 60 min > 0 min. The highest activity in the plasma ORAC assay was observed at 5 min, when the concentrations of vanillin, vanillic acid and protocatechic acid were high.

4. Discussion

As mentioned in the Introduction, vanillin has antimutagenic properties. Recently, it has also been reported that vanillin suppressed the metastatic potential of human cancer cells through PI3K inhibition

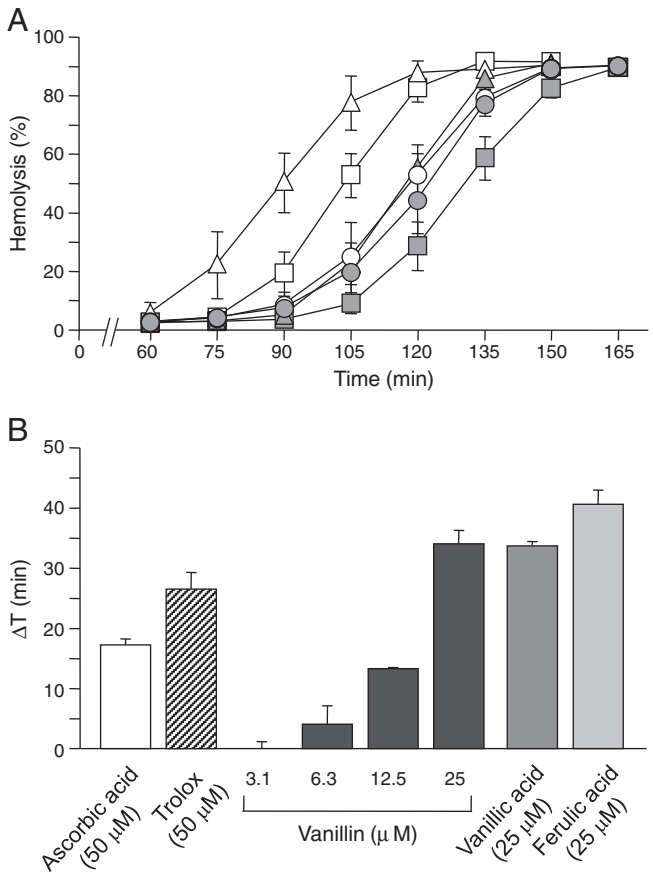


Fig. 7. OxHLIA for vanillin, vanillic acid, ferulic acid, ascorbic acid and Trolox. (A) Sheep erythrocytes at 0.7% (v/v) suspension in PBS were incubated with 40 mM of AAPH in the absence (△) and in the presence of vanillin (25 μM, ●), vanillic acid (25 μM, ○), ferulic acid (25 μM, ■), ascorbic acid (50 μM, □) or Trolox (50 μM, ▲) at 37 °C for 165 min with shaking. Each value is the mean ± SD of five separate experiments. The absence of an SD bar means that the SD bar is within the symbol. (B) Sheep erythrocytes at 0.7% (v/v) suspension in PBS were incubated with 40 mM of AAPH with the indicated concentration of vanillin, vanillic acid, ferulic acid, ascorbic acid or Trolox at 37 °C for 165 min with shaking. Results are expressed as delayed times of hemolysis (ΔT) defined in the text. Each value is the mean + SD of three separate experiments.

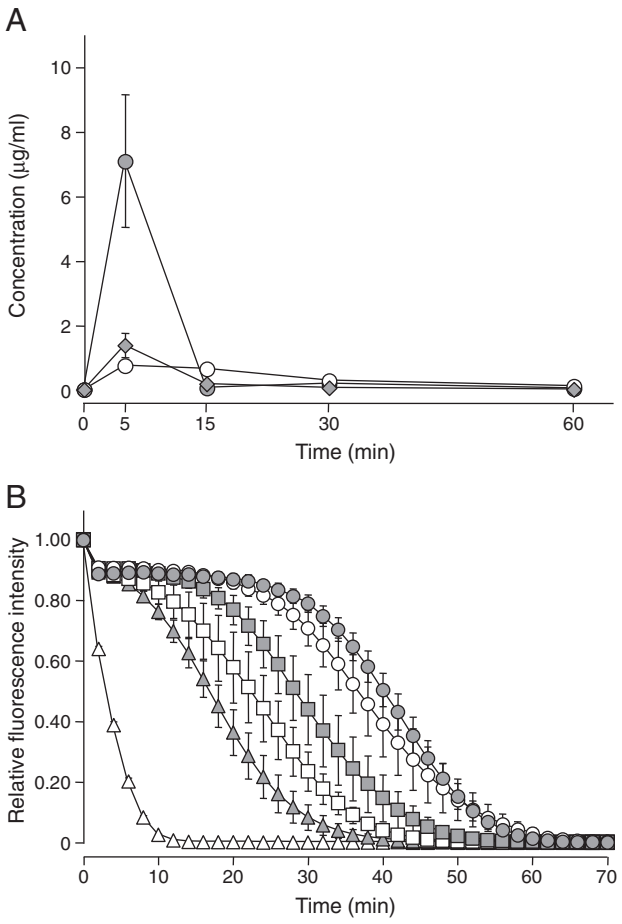


Fig. 8. Contents of vanillin and its metabolites and total antioxidant capacity in plasma after an oral administration of vanillin to mice. Male ICR mice were orally administrated a single dose of vanillin (100 mg/kg). At the indicated times, plasma samples were collected and subjected to HPLC analysis and plasma ORAC assay. (A) Time-course profiles of plasma vanillin (●), vanillic acid (○) and protocatechuic acid (◆) contents were investigated by HPLC analysis. Each value is the mean ± SEM of five separate experiments. (B) In the absence (△) and presence of plasma at 0 min (▲), 5 min (●), 15 min (○), 30 min (■), and 60 min (□), an ORAC assay was carried out. Changes in fluorescence intensity of fluorescein were monitored. Each value is the mean ± SEM of five separate experiments. The absence of an SEM bar means that the SEM bar is within the symbol.

and inhibited angiogenesis in a chick chorioallantoic membrane assay [32], that vanillin improved and prevented trinitrobenzene sulfonic acid-induced colitis in mice [33], that vanillin covalently bound with sickle hemoglobin and inhibited cell sickling [34], and that vanillin exhibited analgesic effects in a mouse [35] or a rat [36] model. Therefore, vanillin with multifunctionality seems to be a beneficial compound for human health. Based on the results of the present study, we propose the addition of antioxidant capacity to the multi-functionality of vanillin.

Vanillin showed stronger activity than did ascorbic acid and Trolox in the ABTS•$^+$-scavenging assay but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays (Fig. 2). Vanillin slowly and continuously reacted with ABTS•$^+$ over a period of 120 min (Fig. 2C). From the EC_{50} value, it is estimated that 1 mol of vanillin scavenged 2.6 mol of ABTS•$^+$ in 120 min of reaction. It is thought that the ABTS•$^+$-scavenging reaction will occur over a more extended time period, since a small amount of vanillin still remained in the reaction mixture after 120 min (Fig. 3D). However, one molecule of vanillin has only one oxidizable phenolic hydroxyl group. There are several findings that are helpful in predicting the radical-scavenging mechanism of vanillin. It has been reported that a 2-pyrone compound [37,38] or AA-2G [24] with one oxidizable –OH group scavenged more than one equivalent of DPPH radical *via* an adduct formation with the radical and that an oxidative dimer formation contributed to the total radical-scavenging ability of protocatechuic esters [39]. Hence, we assumed that vanillin also reacts with radicals *via* adduct formation or self-dimerization.

To predict the radical-scavenging mechanism of vanillin, we tried to isolate reaction products 1 and 2 from reaction mixture of vanillin and ABTS•$^+$ (Fig. 3). The reaction product 2 was purified and identified to be divanillin. Divanillin is a natural product found in a commercial vanilla extract and in cured vanilla beans [40]. The reaction product 1 could not be purified. The reaction product 1 showed a very broad peak compared to that of divanillin by HPLC analysis (Fig. 3) and several spots besides divanillin were observed in the reaction mixture by TLC analysis (data not shown). These data suggested that the reaction product 1 was composed of several compounds. The peaks of vanillin (from 33.0 ± 3.6 μM at 1 min to 5.9 ± 1.0 μM at 120 min) and divanillin (from 4.0 ± 0.1 μM at 1 min to 1.6 ± 0.1 μM at 120 min) decreased with time, whereas the peak of 1 slowly increased. The reaction stoichiometry of divanillin against ABTS•$^+$ was 3.0 at 120 min (data not shown). These results suggested that vanillin scavenged ABTS•$^+$ to generate divanillin at an early stage of the reaction and that divanillin subsequently reacted with additional ABTS•$^+$ to give several reaction products. If the formation of divanillin is the main reaction route for the ABTS•$^+$-scavenging reaction of vanillin, 2 mol of vanillin will scavenge 2 mol of

$ABTS^{\bullet+}$ to generate 1 mol of divanillin, and 1 mol of divanillin will further react with 3 mol of $ABTS^{\bullet+}$. Thus, the reaction stoichiometry of vanillin is expected to be 2.5. This value agrees fairly well with the actual reaction stoichiometry of 2.6. Therefore, these results indicated that an oxidative self-dimerization contributed to the total radical-scavenging ability of vanillin.

Vanillin showed much stronger antioxidant activity than did ascorbic acid and Trolox in the ORAC assay (Fig. 5) and OxHLIA (Fig. 7). The order of inhibition in the ORAC assay was vanillin > vanillic acid ≥ ferulic acid ≫ Trolox > ascorbic acid, and the order of inhibition in the OxHLIA was ferulic acid (25 μM) > vanillin (25 μM) ≥ vanillic acid (25 μM) ≈ Trolox (50 μM) > ascorbic acid (50 μM). The antioxidant activity of ferulic acid was stronger than that of vanillin and vanillic acid in the OxHLIA but was weaker in the ORAC assay. The ORAC assay and OxHLIA use the same radical source, AAPH-derived peroxyl radicals, and results of these assays are therefore correlated with each other to some extent. Vanillin, vanillic acid and ferulic acid possess the same chemical structures for radical-scavenging reactions. However, the orders of activities of ferulic acid and of vanillin and vanillic acid were opposite between the assays. The ORAC assay and OxHLIA utilize the same "hydrophilic" peroxyl radicals but different oxidizable targets, *i.e.*, a "hydrophilic" fluorescein or "lipophilic" biomembrane of erythrocytes. The microlocalization of each antioxidant in OxHLIA may reflect the result that relatively lipophilic ferulic acid was superior to relatively hydrophilic vanillin and vanillic acid in protection against free radical-induced membrane damage.

Divanillin was found in the reaction mixtures of vanillin with AAPH-derived radicals (Fig. 6), suggesting that the strong activity of vanillin in the ORAC assay and OxHLIA was expressed by a mechanism similar to that in the $ABTS^{\bullet+}$-scavenging assay. At 37 °C, 40 mM of AAPH produces ROO• at a constant rate of 3.3 μM/min [41], and the total amount of AAPH-derived radical formed over a period of 30 min is about 100 μM. In our previous study, 50 μM of ascorbic acid was consumed over a period of 30 min. Hence, the reaction ratio of ascorbic acid against ROO• was 1:2, and this agreed well with the present results showing that ascorbic acid reacts with DPPH radical, galvinoxyl radical and $ABTS^{\bullet+}$ at a ratio of 1:2. Vanillin (50 μM) was consumed over a period of 60 min in the presence of 40 mM AAPH (Fig. 6C). The total amount of AAPH-derived radical formed over a period of 60 min is about 200 μM. Thus, 1 mol of vanillin was expected to scavenge about 4 mol of ROO•. The peak of vanillin decreased with time, whereas the peak of divanillin gradually increased over a period of 30 min and had almost disappeared at 60 min. These results indicated that the formation of divanillin contributed to the high reaction stoichiometry against AAPH-derived radicals to result in the unexpectedly strong effect of vanillin.

To investigate the absorption and the antioxidant capacity of vanillin, mice were orally administrated a single dose (100 mg/kg) of vanillin. After oral administration, vanillin and its metabolites, vanillic acid and protocatechic acid, were detected in the plasma. The maximal values of vanillin, vanillic acid and protocatechic acid were 7.1 ± 2.1, 0.75 ± 0.09 and 1.4 ± 0.4 μg/ml, respectively (Fig. 8A). It is noteworthy that the maximal values were reached at 5 min after oral administration. Vanillin was found to metabolize to vanillic acid, protocatechuic acid and vanillyl alcohol in a study using guinea pig liver slices [42]. In a previous pharmacokinetics study, T_{max} and C_{max} were 4.8 min and 2.45 μg/ml after oral administration (100 mg/kg) of vanillin to rats [34]. Our results, except for the absence of vanillyl alcohol as a metabolite, were in reasonable agreement with the results of those previous studies. The highest activity in the plasma ORAC assay was observed at 5 min, when the concentrations of vanillin and its metabolites were highest (Fig. 8B). Although the concentration of vanillin rapidly decreased until 15 min, the high activity in the plasma ORAC assay remained at 15 min. The antioxidant activity in plasma gradually decreased with time, while vanillin and its metabolites rapidly disappeared in plasma. In general, the total antioxidative capacity of plasma appears to be due to the total activity of a variety of compounds, including ascorbic acid, tocopherol, polyphenols, and possibly other endogenous components. In this experiment, antioxidative enzymes were not considered as endogenous components because of the use of deproteinized plasma samples. The slight discrepancy between the concentrations of vanillin and its metabolites in plasma and the antioxidant activity in plasma seems to result from the reproduction of antioxidants and/or the induction of low molecular endogenous antioxidants by administered vanillin. Other metabolites that were not detectable under the analytical conditions also seem to contribute to the antioxidant activity. These results suggested that vanillin intake directly and indirectly raised the antioxidant activity in plasma.

Vanillin is widely used in foods, beverages, cosmetics, and drugs. Vanillin has been reported to exhibit multifunctional effects such as antimutagenic [2–8], antiangiogenetic [32], anti-colitis [33], anti-sickling [34], and antianalgesic effects [35,36]. However, results of studies on the antioxidant activity of vanillin are not consistent. Concentrations of vanillin used in food and beverage products range widely from 0.3 to 33 mM [1]. The high level of vanillin intake from foods and beverages appears to exert some effects on human health. In this study, we systematically evaluated the antioxidant activity of vanillin using multiple assay systems. Vanillin showed stronger activity than did ascorbic acid and Trolox in the $ABTS^{\bullet+}$-scavenging assay but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays. Vanillin showed much stronger antioxidant activity than did ascorbic acid and Trolox in the ORAC assay and OxHLIA. In the $ABTS^{\bullet+}$-scavenging assay, ORAC assay and OxHLIA, vanillin reacted with radicals *via* a self-dimerization mechanism. The dimerization contributed to the high reaction stoichiometry against $ABTS^{\bullet+}$ and AAPH-derived radicals to result in the strong effect of vanillin. Oral administration of vanillin to mice increased the vanillin concentration and the antioxidant activity in plasma. Therefore, antioxidant activity of vanillin might be more beneficial than has been thought for daily health care.

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