Antioxidant Properties of Ethyl Vanillin in Vitro and in Vivo

Akihiro Tai,† Takeshi Sawano, and Futoshi Yazama

Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023, Japan

Received July 13, 2011; Accepted September 12, 2011; Online Publication, December 7, 2011 [doi:10.1271/bbb.110524]

We systematically evaluated the antioxidant activity of ethyl vanillin, a vanillin analog, as compared with the activities of vanillin and other vanillin analogs using multiple assay systems. Ethyl vanillin and vanillin exerted stronger antioxidant effects than did vanillyl alcohol or vanillic acid in the oxygen radical absorbance capacity (ORAC) assay, although the antioxidant activities of vanillyl alcohol and vanillic acid were clearly superior to those of ethyl vanillin and vanillin in the three model radical assays. The antioxidant activity of ethyl vanillin was much stronger than that of vanillin in the oxidative hemolysis inhibition assay, but was the same as that of vanillin in the ORAC assay. Oral administration of ethyl vanillin to mice increased the concentration of ethyl vanillin, and effectively raised antioxidant activity in the plasma as compared to the effect of vanillin. These data suggest that the antioxidant activity of ethyl vanillin might be more beneficial than has been thought in daily health practice.

Key words: ethyl vanillin; ABTS radical cation; oxygen radical absorbance capacity (ORAC); oxidative hemolysis inhibition assay; plasma antioxidant activity

The association of reactive oxygen species and free radicals with many disease states is now well recognized, and antioxidants have attracted considerable attention.1,2) It has been reported that there are two types of antioxidants that scavenge radicals quickly and quench many radicals, and it has been proposed that reactivity should be assessed on the basis of both reaction rate and stoichiometry.3) and that multiple methods should be used, since the activities of some antioxidants vary depending on the assay method.4–6) In addition, comparative studies using common antioxidants appear to be essential to clarify the biological significance of the activities of samples. We have assessed antioxidant activities with attention to the above-mentioned proposals and points of view. We found that 2-O-α-β-glucopyranosyl-β-ascorbic acid (AA-2G), a stable ascorbic acid derivative, exerted radical-scavenging activity toward unnatural model radicals, including DPPH radical7–10) and ABTS radical cation (ABTS+)9,11) The chemical properties of AA-2G as a radical scavenger were widely different from those of ascorbic acid, in that the reaction rate with these model radicals of AA-2G was much slower, but the long-term radical scavenging ability per molecule of AA-2G was superior to that of ascorbic acid. Recently, we reassessed the antioxidant activity of arbutin using five in vitro assay systems, though arbutin has been reported to possess weak antioxidant activity as compared to its precursor, hydroquinone.12) We found that arbutin exerted strong antioxidant activity, comparable or even superior to that of hydroquinone in the ABTS+ scavenging assay, the ORAC assay, and two cell-based antioxidant assays.

Vanillin is widely used in foods, beverages, cosmetics, and drugs. It has been reported to have multifunctional effects, including antimutagenic13–15) antiangiogenic,16) anti-colitis,17) anti-sickling,18) and anti-analgesic,19) but the results of studies of the antioxidant activity of vanillin are not consistent. In a recent study, we systematically evaluated the antioxidant activity of vanillin using multiple assay systems.20) Vanillin showed stronger activity than did ascorbic acid or Trolox in an ABTS+ scavenging assay, but showed no activity in DPPH radical- or galvinoxyl radical-scavenging assays. It also showed much stronger antioxidant activity than did ascorbic acid or Trolox in an ORAC assay and an oxidative hemolysis inhibition assay (OxHLIA). Oral administration of vanillin to mice increased the vanillin concentration and antioxidant activity in the plasma.

Hence, in this study, we systematically evaluated the antioxidant activity of vanillin analogs using multiple assay systems. First we did assays using model radicals, DPPH radical, galvinoxyl radical, and ABTS+. Then we evaluated the antioxidant activity of vanillin analogs by ORAC assay and OxHLIA using physiologically relevant peroxyl radicals. Unexpectedly, ethyl vanillin, a vanillin analog, showed potent antioxidant activity as compared with the activity of Trolox, used as positive control, and we compared it to the activities of vanillin and other vanillin analogs in several in vitro experiments. Finally, the antioxidant activity of ethyl vanillin was confirmed by oral administration of it to mice.

Materials and Methods

Chemicals. Ethyl vanillin and vanillin were purchased from Nacalai Tesque (Kyoto, Japan). 2,2′-Azobis(2-methylpropionamide) dihydrochloride (AAPH) and heparin were from Wako Pure Chemical Industries (Osaka, Japan). Galvinoxyl free radical, vanillyl alcohol, and vanillic acid were from Tokyo Chemical Industry (Tokyo). Apocynin was from Santa Cruz Biotechnology (Santa Cruz, CA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), H2O2 (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) (Z-BHT) was from Sigma-Aldrich (St. Louis, MO). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical, vanillyl alcohol, and vanillic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). 3,5-Dimethylpyrazine was used as a positive control (Kanto Chemical Co., Inc., Tokyo, Japan). All other chemicals were of reagent grade.

† To whom correspondence should be addressed. Fax: +81-824-74-1779; E-mail: atai@pu-hiroshima.ac.jp
of ethyl vanillin- and of vanillin-related compounds was achieved by isocratic elution from an InertSIL ODS-3 column (4.6 i.d. x 250 mm, 5 μm, GL Sciences, Tokyo) kept at 40°C with MeOH/H₂O/acetic acid (40:59:1, v/v) at a flow rate of 0.7 mL/min. The absorbance at 280 nm was monitored. For the plasma ORAC assay, 70 μL of the resulting plasma was deproteinized by adding 140 μL of acetone/H₂O/acetic acid (70:29:5.0, v/v). The mixture was centrifuged at 10,000 x 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 25-fold dilution for the ORAC assay. This assay was carried out under the above-described conditions. The net AUC was calculated by a previous method.21

Results and Discussion

DPH radical-, galvinoxyl radical-, and ABTS⁺-scavenging activities of vanillin analogs

Vanillin showed strong radical-scavenging activity in the ABTS⁺-scavenging assay, but no activity in the DPH radical- and galvinoxyl radical-scavenging assays.20 We evaluated the antioxidant activity of vanillin analogs using model radicals, DPH radical, galvinoxyl radical, and ABTS⁺. Their characteristic colors disappear when they are quenched, and so the decrease in these radicals can easily be monitored with a spectrometer.23,24 We assessed the DPH radical-, galvinoxyl radical-, and ABTS⁺-scavenging activities of vanillin, vanillyl alcohol, vanillic acid, ethyl vanillin, and apocynin in buffered solutions at pH 6, and compared them with those of Trolox, a standard antioxidant. The chemical structures of these compounds are shown in Fig. 1. Trolox showed nearly the same reaction profiles in the three assays (Fig. 2); 20 μM of Trolox rapidly quenched about 40 μM of DPH radical, galvinoxyl radical, and ABTS⁺ within 5 min. Thus their reaction stoichiometries (the number of radical molecules reduced by one molecule of antioxidant) were about 2. This was consistent with our previous studies.20 Vanillin, ethyl vanillin, and apocynin scavenged little or no DPH radical or galvinoxyl radical, while vanillyl alcohol and vanillic acid continuously quenched both radicals throughout the experimental period (Fig. 2A, B). In the ABTS⁺ assay, vanillin and its analogs showed stronger radical-scavenging activity than did Trolox (Fig. 2C). It has been reported that vanillyl alcohol and vanillic acid exerted antioxidant activity.25 Ethyl vanillin and apocynin reacted with ABTS⁺ in a manner similar to that of vanillin. Their reactions proceeded slowly and continuously for 120 min. It is

Fig. 1. Chemical Structures of Vanillin, Vanillyl Alcohol, Vanillic Acid, Ethyl Vanillin, Apocynin, and Trolox.
Ethyl vanillin ( ), apocynin ( ), vanillic acid ( ), vanillyl alcohol ( ), Trolox ( ), 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 68 min. Changes in the fluorescence intensity of fluorescein were monitored. Values are the mean ± SD for triplicate experiments. B, Sheep erythrocytes at 0.7% v/v suspension in PBS were incubated with 40 mM of AAPH in the absence ( ) and the presence of ethyl vanillin ( , 25 µM), apocynin ( , 25 µM), vanillic acid ( , 25 µM), vanillyl alcohol ( , 25 µM), vanillin ( , 25 µM), or Trolox ( , 50 µM) at 37 °C for 180 min with shaking. Values are the mean ± SD for three separate experiments.

**Fig. 3.** ORAC Assay (A) and OxHLIA (B) for Ethyl Vanillin, Apocynin, Vanillic Acid, Vanillyl Alcohol, Vanillin, and Trolox.

A, Reaction mixtures containing ethyl vanillin ( ), apocynin ( ), vanillic acid ( ), vanillyl alcohol ( ), Trolox ( ), 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 68 min. Changes in the fluorescence intensity of fluorescein were monitored. Values are the mean ± SD for triplicate experiments. B, Sheep erythrocytes at 0.7% v/v suspension in PBS were incubated with 40 mM of AAPH in the absence ( ) and the presence of ethyl vanillin ( , 25 µM), apocynin ( , 25 µM), vanillic acid ( , 25 µM), vanillyl alcohol ( , 25 µM), vanillin ( , 25 µM), or Trolox ( , 50 µM) at 37 °C for 180 min with shaking. Values are the mean ± SD for three separate experiments.

**ORAC assay**

Ethyl vanillin and apocynin as well as vanillin showed stronger activity than did 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 efficacies of ethyl vanillin and apocynin in more physiologically relevant assay systems, since 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 peroxyl radical that mimics lipid peroxyl radicals involved in the lipid peroxidation chain reaction in vivo. Inhibition of peroxyl radical-induced oxidation of a fluorescent probe, fluorescein, by antioxidants has been serially monitored. The order of inhibition was ethyl vanillin ≈ apocynin ≈ vanillin > vanillic acid ≥ vanillyl alcohol ≥ Trolox (Fig. 3A). Unexpectedly, the results indicated that ethyl vanillin and apocynin as well as vanillin exerted stronger antioxidant effects than vanillyl alcohol and vanillic acid in the ORAC assay, although the antioxidant activities of vanillyl alcohol and vanillic acid were far superior to those of ethyl vanillin, apocynin, and vanillin in the three model radical assays (Fig. 2).

**Oxidative hemolysis inhibition assay (OxHLIA)**

OxHLIA is a cell-based antioxidant assay using the same radical source as that used in the ORAC assay. Oxidation of erythrocyte membranes by an AAPH-derived peroxyl radical induces the oxidation of lipids...
and proteins and eventually causes hemolysis, and this hemolysis was inhibited by each antioxidant in turn. The order of inhibition was ethyl vanillin (25 μM) ≈ apocynin (25 μM) ≫ vanillin (25 μM) ≈ vanillic acid (25 μM) ≈ vanillyl alcohol (25 μM) ≈ Trolox (50 μM) (Fig. 3B), slightly different from that observed in the ORAC assay (Fig. 3A). The antioxidant activities of ethyl vanillin and apocynin were much stronger than that of vanillin in the OxHLIA but the same as that of vanillin 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 expected to be correlated with each other to some extent. Vanillin, ethyl vanillin, and apocynin have similar chemical structures for radical-scavenging reactions. However, the order of activities of vanillin, ethyl vanillin, and apocynin was different from that in the ORAC. The ORAC assay and OxHLIA utilize the same hydrophilic peroxyl radicals but different oxidizable targets, viz., a hydrophilic fluorescein or a lipophilic biomembrane of erythrocytes. The micro-localizations of the antioxidants in OxHLIA might reflect the result that relatively lipophilic ethyl vanillin and apocynin were superior to relatively hydrophilic vanillin in protection against free radical-induced membrane damage. It has been reported that apocynin promoted the oxidation of glutathione, the sulphhydryl groups in ovalbumin, and NADPH under oxidative conditions.30) These results suggest that ethyl vanillin can be a potent antioxidant in vanillin analogs.

Contents of ethyl vanillin- and vanillin-related compounds and antioxidant capacity in mouse plasma

In some in vitro experiments, ethyl vanillin showed potent antioxidant activity as compared with that of Trolox used as positive control and compared with the activities of known antioxidants, vanillin, vanillyl alcohol, and vanillic acid. To determine the absorption and antioxidant capacity of ethyl vanillin, a single dose (32.7 mg/kg) of ethyl vanillin was administered orally to mice. An equimolar amount (30.0 mg/kg) of vanillin was also administered. After 5, 15, and 30 min, plasma samples were prepared and subjected to HPLC analysis and plasma ORAC assay.

Figure 4A shows typical chromatograms of the plasma 5 min after oral administration of ethyl vanillin and of vanillin. Figure 4B and C shows time-course plots of the plasma concentrations of the ethyl vanillin-and vanillin-related compounds after oral administration. In the ethyl vanillin-treated group, ethyl vanillic acid was detected as a metabolite of ethyl vanillin. The level of ethyl vanillic acid reached a maximum (8.7 μM) at 5 min, and then gradually decreased. In contrast, ethyl vanillin was observed in trace amounts. In the vanillin-treated group, vanillin and its metabolite vanillic acid were detected. The concentration of vanillin reached a maximum (0.5 μM) at 5 min, and then rapidly decreased until 15 min. On the other hand, the level of vanillic acid reached a maximum (1.5 μM) at 5 min, remained at that level until 15 min had elapsed, and then rapidly decreased. In our previous study, vanillin was detected as a major compound following oral administration of vanillin (100 mg/kg).20) It is thought that the difference in the major compound in our previous study was caused by the difference in dosage. It is noteworthy that the maximum values of ethyl vanillin- and vanillin-related compounds were reached 5 min after oral administration of ethyl vanillin and of vanillin. The total amount of the intact form and its metabolite in the ethyl vanillin-treated group was larger than that in the vanillin-treated group. Figure 4D shows ORAC activities in the plasma at 5, 15, and 30 min after oral administration of ethyl vanillin and at 5 min after oral administration of vanillin, ORAC assay was carried out. Changes in the fluorescence intensity of fluorescein were monitored. Values are the mean ± SEM for five separate experiments.

Fig. 4. Contents of Ethyl Vanillin- and Vanillin-Related Compounds and Total Antioxidant Capacity in the Plasma after Oral Administration of Ethyl Vanillin or Vanillin to Mice.

Male ICR mice were orally administered a single dose of ethyl vanillin (32.7 mg/kg) or vanillin (30.0 mg/kg). At the indicated times, plasma samples were collected and subjected to HPLC analysis and plasma ORAC assay. A, HPLC chromatograms of the plasma at 5 min after oral administration of ethyl vanillin or vanillin. Peaks: EVA, ethyl vanillic acid; EVN, ethyl vanillin; VA, vanillic acid; VN, vanillin. B, Time-course profiles of plasma ethyl vanillin (■) and ethyl vanillic acid (▲). The contents were investigated by HPLC. C, Time-course profiles of plasma vanillin (○) and vanillic acid (△) contents were investigated by HPLC. D, In the absence (□) of plasma and in the presence of plasma at 0 min (△), 5 min (■), 15 min (▲), and 30 min (○) after oral administration of ethyl vanillin and at 5 min (○) after oral administration of vanillin, ORAC assay was carried out. Changes in the fluorescence intensity of fluorescein were monitored. Values are the mean ± SEM for five separate experiments.
ethyl vanilic acid (8.7 μM) was exogenously added was approximately the same as that (net AUC, 40.6 ± 3.1, n = 5) of the plasma at 5 min after oral administration of ethyl vanillin, suggesting that ethyl vanilic acid, a metabolite of ethyl vanillin, contributes to the in vivo antioxidant activity. Thus, these results indicate that oral administration of ethyl vanillin to the mice increased the concentration of ethyl vanilic acid and effectively raised the antioxidant activity in the plasma as compared to the effect of vanillin.

Ethyl vanillin and vanillin present in vanilla products as major phenolic constituents are widely used in foods and beverages. Vanillin has been reported to exhibit multifunctional effects, including antimutagenic, antiangiogenic, anti-colitis, anti-sickling, and antinociceptive properties. Recently, we proposed the addition of antioxidant capacity to the multifunctionality of vanillin, but the pharmacological action of ethyl vanillin has been little reported. To our knowledge, there is only one report on its anti-angiogenic, anti-inflammatory, and antinociceptive properties. In the present study, we found a potent antioxidant effect of ethyl vanillin as compared with the effects of vanillin and other vanillin analogs using multiple assay systems. Ethyl vanillin and vanillin had stronger antioxidant effects than vanillyl alcohol or vanillic acid in the ORAC assay, although the antioxidant activities of vanillyl alcohol and vanillic acid were clearly superior to those of ethyl vanillin and vanillin in the three model radical assays. The antioxidant activity of ethyl vanillin was much stronger than that of vanillin in the OxHILA, but was the same as that of vanillin in the ORAC assay. Oral administration of ethyl vanillin to mice increased the concentration of ethyl vanillic acid, a metabolite of ethyl vanillin, and effectively raised the antioxidant activity in the plasma as compared with vanillin. Therefore, antioxidant activity of ethyl vanillin might be more beneficial than is thought in daily health practice.

References