Antioxidative Properties of Vanillic Acid Esters in Multiple Antioxidant Assays

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Received September 20, 2011; Accepted November 8, 2011; Online Publication, February 7, 2012
[doi:10.1271/bbb.110700]

The antioxidative properties of vanillic acid esters were systematically evaluated by multiple assays to compare with the well-known antioxidants, vanillic acid and Trolox. We first performed assays with the model radicals, DPPH, galvinoxyl and ABTS cation (ABTS**) types. Methyl vanillate, ethyl vanillate and butyl vanillate showed stronger activity than Trolox in the ABTS**-scavenging assay, but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays. In contrast, vanillic acid could quench the three radicals. We then evaluated their antioxidative activities by an ORAC assay and an oxidative hemolysis inhibition assay (OxHLIA), using physiologically relevant peroxyl radicals. Vanillic acid esters and vanillic acid exerted much stronger activity than Trolox in the ORAC assay and OxHLIA. The antioxidative activity by OxHLIA was strongly correlated to the lipophilicity of vanillic acid and its esters. These results indicate that the protective effect of vanillic acid esters against free radical-induced biomembrane damage increased with increasing lipophilicity.

Key words: vanillic acid ester; antioxidant; ABTS radical cation; ORAC; oxidative hemolysis inhibition assay

Two types of antioxidants have been reported that quickly scavenge radicals and quench many radicals. It has been proposed that reactivity should be assessed on the basis of both the reaction rate and stoichiometry,1) and that multiple methods should be used, since the activities of some antioxidants vary according to the assay method.1–4) Comparative studies using common antioxidants also appear to be essential to clarify the biological significance of the activities of samples. We have assessed antioxidative activities according to these principles. We found that 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), a stable ascorbic acid derivative, exerted radical-scavenging activity toward unnatural model radicals, including the DPPH radical1–8) and ABTS cation (ABTS**) types.7,9) The chemical properties of AA-2G as a radical scavenger differed markedly from those of ascorbic acid, in that the reaction rate with this model radical of AA-2G was much slower than that of ascorbic acid, although the long-term radical scavenging ability per molecule of AA-2G was superior to that of ascorbic acid. We also found by using the cell-based oxidative hemolysis inhibition assay (OxHLIA) that the radical-scavenging activity of AA-2G was biologically relevant.10) We have recently reassessed the antioxidative activity of arbutin by using five in vitro assays, although arbutin has been reported to possess weak antioxidative activity when compared to its precursor, hydroquinone.11) We found that arbutin exerted strong antioxidative activity comparable or even superior to that of hydroquinone in the ABTS**-scavenging, ORAC, and two cell-based antioxidative assays.

Vanillin (Fig. 1), a compound widely used in foods, beverages, cosmetics and drugs, has been reported to have such multiple functions as antimutagenic,12–18) antiangiogenic,19) anti-colitis,20) anti-sickling,21) and antianalgesic effects.22,23) However, the results of studies on the antioxidative activity of vanillin have not been consistent. We have recently systematically evaluated the antioxidative activity of vanillin by multiple assays.24) Vanillin showed stronger activity than both ascorbic acid and Trolox in the ABTS**-scavenging assay, but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays. Vanillin also showed much stronger antioxidative activity than both ascorbic acid and Trolox in the ORAC assay and OxHLIA. An oral administration of vanillin to mice increased the vanillin concentration and antioxidative activity in the plasma. We have more recently found that ethyl vanillin (Fig. 1) showed stronger antioxidative activity than vanillin in OxHLIA and an in vivo assay, although their activity was almost same in the ABTS**-scavenging and ORAC assays.25) These results suggest that relatively lipophilic ethyl vanillin had stronger antioxidative activity than relatively hydrophilic vanillin in OxHLIA and the in vivo assay. Vanillic acid showed superior radical-scavenging activity to either ethyl vanillin or vanillin in the three model radical assays and also had antioxidative activity in the ORAC assay and OxHLIA.25) The antioxidative activity of vanillic acid esters was therefore expected to be greater than that of vanillic acid in several antioxidative assays, especially by OxHLIA.

Hence, we systematically evaluated in this study the antioxidative properties of vanillic acid and its esters by multiple assays. We first performed assays by using the model radicals, DPPH radical, galvinoxyl radical, and ABTS**. We then evaluated the antioxidative activity of the vanillates by an ORAC assay and OxHLIA, using...
physiologically relevant peroxyl radicals. We finally identified a relationship between the antioxidative activity and lipophilicity of vanillic acid and its esters in OxHLIA.

Materials and Methods

Chemicals. Ethyl vanillate and 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The galvinoxyl free radical, methyl vanillate, and vanillic acid were obtained from Tokyo Chemical Industry (Tokyo, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), \( \text{H}_2\text{O}_2 \) (3%), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich Chemicals (Milwaukee, WI, USA). Ethyl vanillate, ethyl vanillate, and butyl vanillate were separated by isocratic elution from an Inertsil ODS-3 column (250 mm × 4.6 mm i.d.) containing 1% acetic acid at a flow rate of 0.7 mL/min. The various antioxidants were dissolved in DMSO, and the DMSO solution was mixed with an antioxidant (20 μM) in a 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 in ABTS** scavenging activity was assessed by measuring the absorbance at 530 nm with a spectrophotometer.

**OxHLIA** assay. The OxHLIA assay was carried out as previously described. Briefly, sheep erythrocytes suspended at a concentration of 0.7% (v/v) in phosphate-buffered saline (PBS) were incubated at 37 °C with 40 mM of AAPH in the presence of an antioxidant (25 or 50 μM) while shaking. The various antioxidants were dissolved in DMSO, and the DMSO solution was added at 100 μM to the mixture. The decrease in the absorbance at 524 nm was monitored.

**Distribution of vanillates in the sheep erythrocytes.** Vanillic acid, methyl vanillate, ethyl vanillate, and butyl vanillate (25 μM) dissolved in DMSO were diluted with PBS to give 500 μL of a 500 μM solution. Five hundred μL of sheep erythrocytes suspended at a concentration of 14% (v/v) in PBS was added to the resulting solution, and the mixture was placed in an M-BR-022 thermoregulated shaker (Taitec, Saitama, Japan) and stirred at 1,100 r/min for 30 min at 37 °C. The mixture was centrifuged at 10,000 g for 2 min after the incubation, and the resulting supernatant was removed by pipetting. The erythrocyte pellet was washed twice with 500 μL of PBS after centrifugation, and the washed erythrocyte pellet was lysed in 500 μL of water. The lysate was extracted with 1.0 mL of EtOAc, and the mixture was centrifuged at 10,000 g for 2 min; 800 μL of the ethyl acetate phase was then removed and concentrated to dryness in a stream of air. The resulting residue was added 80 μL of the HPLC solvent. The solution was directly subjected to an HPLC analysis to evaluate the content of each compound, using a system consisting of an L-7100 pump, L-7420 UV-VIS detector, L-7300 column oven, and D-2500 chromato-integrator (Hitachi High-Technology, Tokyo, Japan). Vanillic acid, methyl vanillate, ethyl vanillate, and butyl vanillate were separated by isocratic elution from an Inertsil ODS-3 column (4.6 i.d. × 250 mm, 5 μm; GL Sciences, Tokyo, Japan) kept at 40 °C with 40%, 50%, or 60% MeOH-H\(_2\)O containing 1% acetic acid at a flow rate of 0.7 mL/min. The absorbance at 260 nm was monitored.

**Results**

**DPPH radical-, galvinoxyl radical-, and ABTS**** scavenging activities**

DPPH radical, galvinoxyl radical, and ABTS** scavenging activities are relatively stable radicals. Their characteristic absorption maxima in the visible region disappear when they are quenched, enabling the decrease of these radicals to be easily monitored by a spectrometer. We assessed the DPPH radical-, galvinoxyl radical-, and ABTS** scavenging activities of the vanillic acid esters in buffered solutions at pH 6 for 120 min and compared them with those of vanillic acid and Trolox. Three esters of differing polarity, methyl vanillate, ethyl vanillate, and butyl vanillate, were selected to evaluate the antioxidative properties. The chemical structures of these compounds are shown in Fig. 1. Trolox showed nearly the same reaction profiles in the three assays (Fig. 2), i.e., 20 μM of the antioxidant rapidly quenched about 40 μM of the DPPH radical, galvinoxyl radical and ABTS** scavenging activities.
within 5 min. The reaction stoichiometry of each (the number of radical molecules reduced by one molecule of antioxidant) was therefore about 2, this being consistent with the result of our previous studies.7,24) Unexpectedly, the vanillic acid esters scavenged little or none of the DPPH radical or galvinoxyl radical, while vanillic acid continuously quenched both radicals throughout the experimental period (Fig. 2A and B). The vanillic acid esters and vanillic acid showed significant radical-scavenging activity in the ABTS+/- scavenging reactions of vanillic acid, methyl vanillate, ethyl vanillate, butyl vanillate, and Trolox.

Vanillic acid (▲), methyl vanillate (▲), ethyl vanillate (○), butyl vanillate (△), Trolox (□) (20 μM each) or the control (○), and the DPPH radical or galvinoxyl radical (100 μM) were incubated at room temperature in a 60% ethanol/40% citrate buffer (10 mM, pH 6). The sample or control and ABTS**+/ABTS+/C15+ (100 μM) were incubated at room temperature in a 2% EtOH/citrate buffer (50 mM, pH 6). The changes in the remaining radicals were measured at the indicated times. Each value is the mean ± SD of three separate experiments. Absence of the SD bar means that the bar is within the symbol.

The reaction between the vanillic acid esters and ABTS**+ proceeded slowly and continuously for 120 min. The reaction profile for each vanillic acid ester with ABTS**+ was nearly the same, the vanillic acid esters (20 μM) having quenched about 20 μM of ABTS**+ after 5 min and about 50 μM after 120 min. The reaction stoichiometry of each was therefore about 1 after 5 min and about 2.5 after 120 min. These results indicate that methyl vanillate, ethyl vanillate and butyl vanillate showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays, but showed stronger activity than Trolox in the ABTS**+/-scavenging assay.

ORAC assay

The vanillic acid esters showed stronger activity than Trolox in the ABTS**+/-scavenging assay, but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays. This difference led us to assess the antioxidative efficacy of the vanillic acid esters in more physiologically relevant assays, because the DPPH radical, galvinoxyl radical and ABTS**+ are unnatural radical species that are not present in the human body. The ORAC assay utilizes an AAPH-derived peroxyl radical which mimics the lipid peroxyl radicals involved in the lipid peroxidation chain reaction in vivo. Inhibition by the antioxidants of the peroxyl radical-induced oxidation of the fluorescent probe, fluorescein, is serially monitored.28)

Methyl vanillate, ethyl vanillate, and butyl vanillate had strong antioxidative activities in the ORAC assay, the degree of inhibition being methyl vanillate ≥ ethyl vanillate ≥ butyl vanillate ≥ vanillic acid ≥ Trolox (Fig. 3). The inhibition profile for fluorescence decay by the vanillic acid esters was slightly different from that by vanillic acid. The vanillic acid esters and Trolox completely inhibited the loss of fluorescence in the early phase of the reaction, the fluorescence rapidly decaying after respective lag times of about 32 min and 12 min. Vanillic acid continued its partial inhibition for a longer period, the profiles of the fluorescence decay curves for the vanillic acid esters and vanillic acid therefore being a little different. In contrast, the total extent of inhibition by an antioxidant in the ORAC assay is expressed by net AUC which is obtained by subtracting the area under the blank curve from the area under the sample curve. The net AUC values for vanillic acid, methyl vanillate, ethyl vanillate, butyl vanillate, and Trolox were 24.6 ± 0.4, 32.7 ± 0.4, 30.1 ± 0.4, 27.1 ± 1.1, and 4.6 ± 0.0, respectively (mean ± SD of triplicate experiments). The overall antioxidative activity of the vanillic acid esters in the ORAC assay was therefore in roughly the same range as that of vanillic acid.
Sheep erythrocytes as a 0.7% (v/v) suspension in PBS were incubated with 40 mM of AAPH in the absence (□) and presence of vanillic acid (25 μM, △), methyl vanillate (25 μM, ○), ethyl vanillate (25 μM, ●), butyl vanillate (25 μM, •) or Trolox (50 μM, ▲) at 37 °C for 225 min while shaking. Each value is the mean ± SD of three separate experiments. Absence of the SD bar means that the bar is within the symbol.

**Figure 4. OxHLIA for Vanillic Acid, Methyl Vanillate, Ethyl Vanillate, Butyl Vanillate, and Trolox.**

**OxHLIA** is a cell-based antioxidative assay using the same radical source as that used for the ORAC assay. The oxidation of erythrocyte membranes by an AAPH-derived peroxyl radical induces the oxidation of lipids and proteins and eventually causes hemolysis, this hemolysis being inhibited by each antioxidant (Fig. 4). The degree of inhibition was butyl vanillate (25 μM) > ethyl vanillate (25 μM) > methyl vanillate (25 μM) > vanillic acid (25 μM) ≈ Trolox (50 μM), which is substantially different from that obtained by the ORAC assay (Fig. 3). The results are expressed as the delayed time for hemolysis (ΔT) calculated by the following equation:

\[ ΔT = HT_{50}(sample) - HT_{50}(control) \]

where HT50 is time to reach 50% hemolysis. The respective ΔT values for vanillic acid (25 μM), methyl vanillate (25 μM), ethyl vanillate (25 μM), butyl vanillate (25 μM), and Trolox (50 μM) were 31.9 ± 2.3, 65.9 ± 2.0, 79.7 ± 2.4, 110.7 ± 3.7, and 31.0 ± 2.0 min (mean ± SD of three separate experiments). These results suggest that the inhibitory effects on hemolysis of vanillic acid and its esters were lipophilicity-dependent.

Erythrocytes (7.0% v/v in PBS, 2.0 × 10^9 cells/mL) were incubated with various vanillates (250 μM) at 37 °C for 30 min, and the distribution of the vanillates in sheep erythrocytes was determined by HPLC. The values are expressed as moles per total cell number in 1 mL. The respective distribution amounts for vanillic acid, methyl vanillate, ethyl vanillate, and butyl vanillate were 0.2 ± 0.0, 2.1 ± 0.4, 2.9 ± 0.3, and 18.1 ± 1.3 nmol/2.0 × 10^9 cells (mean ± SD of three separate experiments). Outstanding linearity was obtained for the relationship between the distribution amount (y) and the ΔT value (x) on a semi-logarithmic plot (Fig. 5).

**Discussion**

The antioxidative activity of vanillic acid is well-known from *in vitro* experiments. Unexpectedly, methyl vanillate, ethyl vanillate and butyl vanillate showed significantly less scavenging activity against the DPPH radical, galvinoxyl radical, and ABTS**⁺⁺ than vanillic acid did (Fig. 2). Vanillic acid and its esters have a carboxyl group and carboxylate ester groups that are generally considered as being electron-withdrawing. However, since the carboxyl group of vanillic acid was deprotonated at pH 6 in the three radical-scavenging assays, the resulting carboxylate group appears to have shown an electron-donating effect. It thus seems that the radical-scavenging ability depended on the electron-donating capability of the substituted groups. Methyl vanillate, ethyl vanillate and butyl vanillate showed stronger activity than Trolox in the ABTS**⁺⁺-scavenging assay, but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays. The vanillic acid esters slowly and continuously reacted with ABTS**⁺⁺ over a period of 120 min in the same way (Fig. 2C). It is estimated that one mole of vanillic acid esters scavenged 2.5 moles of ABTS**⁺⁺ in 120 min of the reaction, although one molecule of vanillic acid esters has only one oxidizable phenolic hydroxyl group. It has been reported that a 2-pyrene compound or AA-2G with one oxidizable –OH group scavenged more than one equivalent of the DPPH radical via adduct formation with the radical and that oxidative dimer formation contributed to the total radical scavenging ability of protocatechuic esters or vanillin. We therefore assume that the vanillates reacted with the radicals via self-dimerization in the same manner as vanillin.

Methyl vanillate, ethyl vanillate and butyl vanillate, as well as vanillic acid, had strong antioxidative activities in the ORAC assay (Fig. 3). The degree of inhibition was methyl vanillate ≥ ethyl vanillate ≥ butyl vanillate ≥ vanillic acid ≥ Trolox. The values for net AUC suggest that the overall antioxidative activity of the vanillic acid esters in the ORAC assay was roughly in the same range as that of vanillic acid. The vanillic acid esters showed much stronger antioxidative activity than vanillic acid and Trolox in the OxHLIA assessment (Fig. 4). The degree of inhibition of vanillic acid and its esters by OxHLIA was lipophilicity-dependent as expected from the results of the previous study on vanillin and ethyl vanillin. The ORAC assay and OxHLIA used the same radical source, AAPH-derived peroxyl radicals, and the results of these assays would therefore be correlated with each other to some extent. However, the degree of this ability was not correlated in both assays. The antioxidative activity of...
the vanillic acid esters was stronger than that of vanillic acid by OxHLIA, but was same by the ORAC assay. These results suggest that the reactivity of the AAPH-derived peroxyl radicals and compounds was not the reason for the difference in antioxidative activity between the ORAC assay and OxHLIA.

Vanillic acid and its esters possess similar chemical structures for the radical-scavenging reactions. The ORAC assay and OxHLIA utilized the same hydrophilic peroxyl radicals, but different oxidizable targets; viz., a hydrophilic fluorescein or a lipophilic biomembrane of erythrocytes. The micro-localization of the antioxidants in OxHLIA might have reflected the result that relatively lipophilic vanillate was superior to relatively hydrophilic vanillic acid in protecting against free radical-induced membrane damage. Figure 5 shows outstanding linearity between the distribution amount in erythrocytes and the ΔT value by OxHLIA. These results suggest that vanillic acid and its esters exerted lipophilicity-dependent antioxidative activity in the OxHLIA assessment. We systematically evaluated the antioxidative properties of vanillic acid and its esters in this study by using multiple assays. Methyl vanillate, ethyl vanillate and butyl vanillate showed stronger activity than Trolox in the ABTS⁺-scavenging assay, but showed no activity in the DPPH radical- and galvinoxyl radical-scavenging assays. In contrast, vanillic acid could quench all three radicals. The vanillic acid esters and vanillic acid exerted much stronger activity than Trolox in the ORAC assay and OxHLIA assessment. The antioxidative activity by OxHLIA was strongly correlated with the lipophilicity of vanillic acid and its esters. These results indicate that the protective effect of the vanillic acid esters against free radical-induced biomembrane damage increased with increasing lipophilicity. These findings suggest that both the vanillic acid esters and vanillic acid might act as potent antioxidants, although some differences in the antioxidative properties between the esters and the acid were apparent in the multiple antioxidant assays.

Acknowledgments

The authors are grateful to the SC-NMR Laboratory of Okayama University and the MS Laboratory of Faculty of Agriculture at Okayama University.

References