JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Inhibition of Peroxynitrite-Mediated Reactions by Vanillin

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Several neurodegenerative diseases such as Alzeimer's and Parkinson's as well as septic shock and inflammation involve formation of reactive oxygen and nitrogen species that include peroxynitrite (PON). PON can also react with endogenous antioxidants. Therefore, dietary supplementation with antioxidants may help in these diseases. An exogenous antioxidant, vanillin (4-hydroxy-3-methoxybenzaldehyde), used widely as a food flavoring agent, was evaluated for its ability to scavenge PON and inhibit PON-mediated reactions. Nitration of tyrosine by PON was assessed by high-performance liquid chromatography (HPLC). This reaction was inhibited by vanillin. The oxidation of dihydrorhodamine 123 to fluorescent rhodamine 123 was also inhibited by vanillin. The kinetics of reaction between PON and vanillin was studied by stopped-flow technique. The products of this reaction were analyzed by HPLC, and hydroxyvanillin was identified as one of the five products with absorption at 350 nm. These data demonstrate that vanillin effectively scavenges PON in cell-free systems.

KEYWORDS: Vanillin; peroxynitrite; phenoxyl radicals; pulse radiolysis; stopped-flow; antioxidant

INTRODUCTION

Peroxynitrite (PON) is formed in vivo by diffusion-controlled reaction of nitric oxide (NO) with superoxide anions ($O_2^{\bullet -}$) (*1*, *2*). Stimulated macrophages, neutrophils, and endothelial cells are known to produce PON, and its in vivo formation has been shown during pathophysiological conditions (*3*−*6*). It has been implicated in several neurodegenerative diseases such as Alzheimer's and Parkinson's, in ischemia-reperfusion injury, and also in septic shock and inflammation (*7*−*10*). Under physiological conditions, PON reacts with various cellular macromolecules such as DNA, lipids, and proteins, leading to strand breaks, lipid peroxidation, and nitration of amino acids, respectively (*11*−*16*). In addition, PON reacts with different endogenous antioxidants such as ascorbate, α-tocopherol, and thiol-containing molecules, which form the primary defense against oxidative stress, leading to their depletion and reduced availability (*17*−*20*). Hence, exogenous compounds, especially of dietary origin, that are capable of scavenging reactive oxygen/nitrogen species may play a pivotal role in preventing/controlling degenerative diseases.

Vanillin, a plant phenol, is used widely as a food flavoring agent in confectioneries, chocolates, butter, toppings, icings, distilled spirits, etc. (*21*, *22*). Its antioxidant activity has been ascertained using diphenylpicrylhydrazyl radical (DPPH) assay in our laboratory (*23*). Furthermore, it was shown to protect DNA and mitochondrial membrane against oxidative stress in vitro (*24*−*26*). Vanillin displayed antimutagenic activity in both bacteria and mammalian cells (*27*, *28*). It reduced chromosomal damage induced by mitomycin C, hydrogen peroxide, and ionizing radiation in cultured Chinese hamster ovary cells (*28*, *29*). It also inhibited chemically induced hepatocarcinogenesis in rat (*30*, *31*). However, evidence for scavenging of highly reactive nitrogen species (RNS) such as PON by vanillin has not been reported so far.

The amino acid tyrosine has been shown to be susceptible to the action of PON, and the stable end product, 3-nitrotyrosine, is used as a standard oxidative marker. The ability to inhibit nitrotyrosine formation provides a useful assay to screen compounds for PON scavenging ability (*32*, *33*). The other type of reaction of PON with its substrates is oxidation. The inhibition of PON-induced oxidation of dihydrorhodamine 123 to rhodamine 123 is also a widely used assay for antioxidants (*34*). The present studies were, therefore, carried out to assess the ability of vanillin to inhibit PON reactions and to separate and identify the products generated during the reaction. The kinetics of the reaction between PON and vanillin was also monitored by different methods.

MATERIALS AND METHODS

Vanillin, hydroxyvanillin, dihydrorhodamine 123 (DHR123), tyrosine, and nitrotyrosine were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO. Sodium azide, phosphate salts, potassium nitrite, and potassium nitrate of the highest purity available were obtained locally from Glaxo India Ltd., Mumbai. IOLAR grade gases were used.

Synthesis of Peroxynitrite. PON was synthesized by the ozonolysis of alkaline sodium azide solution for 2 h at 0−4 °C as described by

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10.1021/jf030319d CCC: $27.50
Published on Web 12/09/2003

Pryor et al. (*35*). The PON solution thus prepared was stored at −20 °C and used within 3−4 weeks. The concentration of PON was determined by measuring its absorbance at 302 nm, using an extinction coefficient of 1670 M^{-1} cm^{-1}. Solutions were prepared in Nanopure water obtained from a Millipore system.

Tyrosine Nitration Assay. A 50 μL aliquot of PON (500 μM) was added to a solution containing tyrosine (100 μM) in the absence or presence of various concentrations of vanillin in 0.2 M phosphate buffer, pH 7.0, in a final volume of 1 mL (*33*). A high concentration of buffer was used to ensure that the samples remained at pH 7.0 even after the addition of alkaline PON solution. The samples were then analyzed by HPLC using a C18 column. The mobile phase used was 50 mM phosphate buffer, pH 7.0, in 5% acetonitrile at a flow rate of 0.5 mL/min. The amount of 3-nitrotyrosine formed was determined from the HPLC profile of a standard monitored at 430 nm.

Assay of Peroxynitrite-Mediated Oxidation of Dihydrorhodamine 123. The PON-induced oxidation of DHR123 was performed as described by Kooy et al. (*36*). Briefly, PON (final concentration = 10 μM) was added to 50 μM DHR123 in the absence or presence of different concentrations of vanillin in 0.1 M phosphate buffer containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA), pH 7.3, at room temperature. The fluorescence of the oxidized product was measured in a spectrofluorometer (Hitachi F4010) with excitation and emission wavelengths of 500 and 536 nm, respectively. At this wavelength interference from vanillin was not observed.

Kinetic Studies. Stopped-flow experiments were carried out using an SX-18 MV multimixing stopped-flow reaction analyzer from Applied Photo Physics Ltd. It was used in the single mixing mode, and the reaction was monitored using absorption detection. In this, syringe I contained PON in 0.01 M Na_2HPO_4 buffer, pH 8.5 (solution A), and syringe II contained 0.1 M phosphate buffer, pH 7.0, with or without vanillin (solution B). The higher concentration of buffer in syringe II ensured that the pH did not exceed 7.0 after the mixing of solutions A and B. All of the experiments were carried out at room temperature (25 °C). At least three independent runs were used to determine the observed rates at any particular concentration.

HPLC Analysis. PON (final concentration = 0.5 mM) was added to reaction mixtures containing various concentrations of vanillin in phosphate buffer with rapid vortex mixing, and the final pH of the solution was 7.0. The reaction was complete within several seconds after the addition of PON. Standard incubation time was 5 min. This reaction mixture was filtered through a 0.2 μm filter and was separated on a 3.9 × 150 mm reverse phase HPLC column (Nucleosil, C18) using Waters model 515 pumps and an automated gradient controller. Separations were carried out under isocratic conditions using 0.1 M citric acid, pH 2.0, and 15% acetonitrile at a flow rate of 0.5 mL/min with UV detection at 350 nm.

Pulse Radiolysis. Pulse radiolysis experiments were performed using 7 MeV electrons from a linear accelerator. All of the experiments were carried out using a 50 ns pulse with an absorbed dose of 11−12 Gy (*37, 38*). The absorbed dose was measured using aerated thiocyanate dosimeter monitoring $(SCN)_2^{\bullet -}$ at 500 nm (*37*). Radiolysis of water generates reactive radicals such as e^-_{aq}, $^\bullet OH$, and $^\bullet H$ and less reactive molecular species such as $HO_2^\bullet$ and H_2O_2 (*38*). Reactions of hydroxyl radical were studied by irradiating N_2O-saturated aqueous solutions, where e_{aq}^- was quantitatively converted to $^\bullet OH$ radical ($e^-_{aq} + N_2O \rightarrow {}^-OH + {}^\bullet OH + N_2$). $NO_2^\bullet$ radicals were generated by the radiolysis of N_2O-saturated aqueous solutions containing 20 mM sodium nitrite. Under these conditions, reaction of hydroxyl radicals with nitrite ions ($NO_2^- + {}^\bullet OH \rightarrow NO_2^\bullet + OH^-$) produced $NO_2^\bullet$ radicals.

RESULTS AND DISCUSSION

Figure 1a shows the percent inhibition of formation of 3-nitrotyrosine during the reaction of PON with tyrosine in the presence of different concentrations of vanillin. There was a sharp rise in the inhibition of 3-nitrotyrosine formation with increasing concentration of vanillin, reaching ~85% inhibition at 100 μM. At 250 μM vanillin, the formation of 3-nitrotyrosine was completely inhibited (**Figure 1a**). PON can react with both free and protein-bound tyrosine (*39*). Nitration of proteins can potentially affect many key cellular processes such as the phosphorylation of proteins by protein tyrosine kinases crucial for cell proliferation and differentiation (*40*).

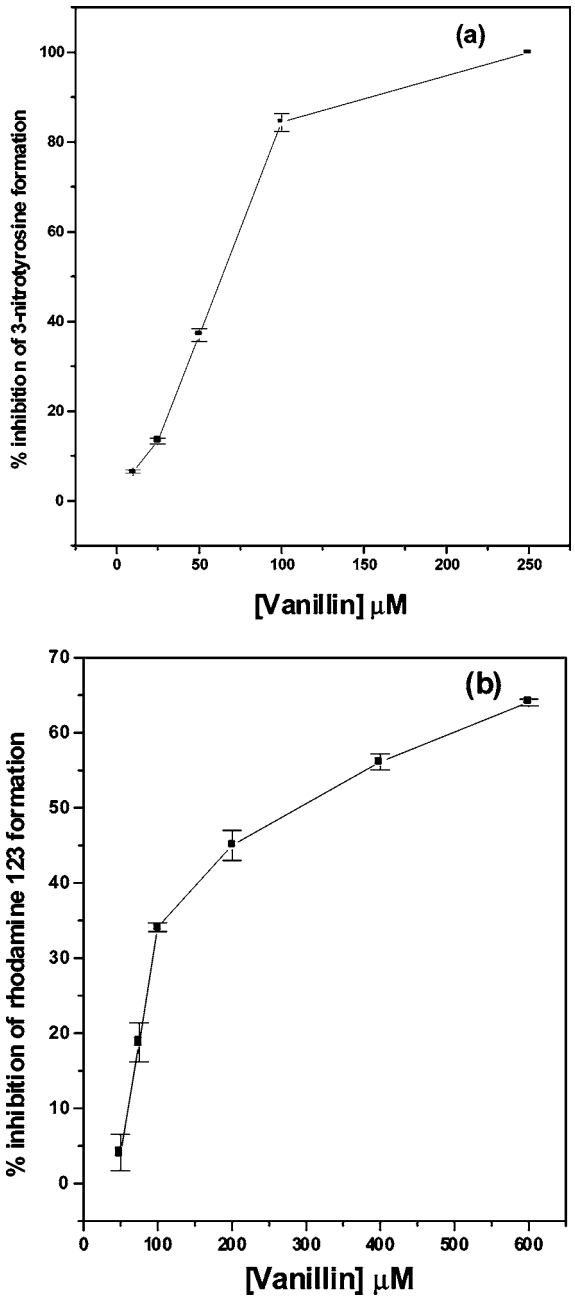


Figure 1. (**a**) Inhibition of PON-induced formation of 3-nitrotyrosine by vanillin. Percent decrease was calculated on the basis of the area under the 3-nitrotyrosine peak detected at 430 nm using HPLC. (**b**) Percent inhibition of PON-mediated oxidation of dihydrorhodamine 123 by vanillin. Percent inhibition was calculated on the basis of fluorescence intensity of rhodamine 123 in the absence of vanillin.

Figure 1b depicts the percent inhibition of PON-induced oxidation of dihydrorhodamine 123 to rhodamine 123 by vanillin. The percent inhibition increased sharply with increasing concentration of vanillin up to 100 μM and slowly thereafter. Fifty percent inhibition was observed at ~300 μM. It also prevented oxidation of DHR123 effectively. DHR123 is an efficient and sensitive dye enabling the detection of submicromolar concentrations of PON (*36*). It has been proposed that by single electron transfer from DHR123 to PON, the dihydrorhodamine radical is formed, which can dismutate to form

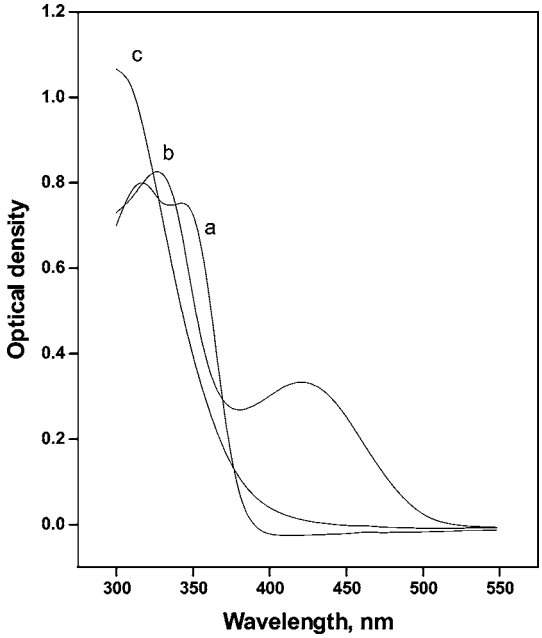


Figure 2. Steady-state absorption spectra of (**a**) 50 μM vanillin at pH 7.0 and (**b**) the product of the reaction of 50 μM vanillin with 500 μM PON at pH 7.0. The absorption spectrum of blank PON is shown for comparison (**c**).

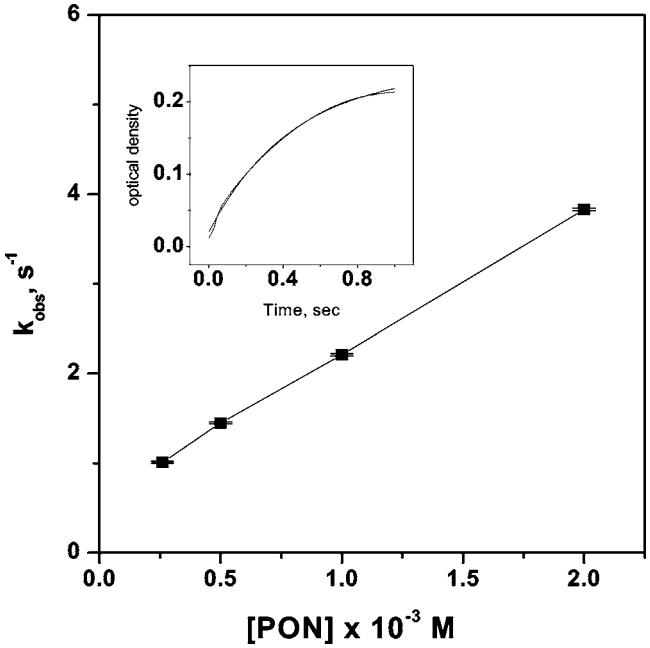


Figure 3. Linear plot showing the variation of observed reaction rate for the formation of the product at 430 nm as a function of PON concentration after mixing with 50 μM vanillin at pH 7.0. (Inset) Formation of the product at 430 nm with time after mixing PON with vanillin.

rhodamine 123 and dihydrorhodamine 123 (*36*). The fluorescent product rhodamine 123 is stable and remains well localized within the cells, making DHR123 a useful probe for the detection and quantification of PON.

Figure 2a shows the absorption spectrum of vanillin at pH 7 in the wavelength region from 300 to 550 nm. The absorption spectrum of the reaction product of PON with vanillin, with a new peak at 430 nm, is shown in **Figure 2b**. For comparison, the absorption spectrum of PON is given in **Figure 2c**. These spectra demonstrated that PON reacted with vanillin to produce a new species having an absorption that is red shifted with respect to the parent.

The rate constant for the reaction of vanillin with PON was determined using a stopped-flow spectrometer. In the absence of vanillin the decay of PON was first-order with a rate constant of 0.52 s^{-1}. This decay could not be followed in the presence of vanillin due to the strong absorption by both vanillin and its products at 302 nm. In the presence of vanillin (50 μM), under the same pH conditions, the absorption of vanillin at 350 nm decreased with time with a simultaneous formation of a new peak between 400 and 430 nm. The rate of formation of product at 430 nm was followed as a function of PON concentration varying from 250 μM to 2 mM by fixing the concentration of vanillin at 50 μM. The rate constant (k_1) for the reaction of PON with vanillin was determined as follows:

$$\text{PON} + \text{VAN} \xrightarrow{k_1} \text{product} \qquad (1)$$

The rate of change in the formation of product (k_{obsd}) was followed over a range of concentration of PON, under pseudo-first-order conditions, where [PON] $\gg$ [VAN]. A linear relationship was used for the formation of the new product at 430 nm with the change in PON concentration as $k_{obsd} = A + k_1$[PON], where A is a constant and k_1 the second-order rate constant of the above reaction, which is obtained from the slope of the linear plot of k_{obsd} versus [PON] (**Figure 3**). The slope gave a bimolecular rate constant (k_1) of 1600 M^{-1} s^{-1}.

The bimolecular rate constant (k_1) of PON with vanillin was also estimated independently using the fluorescence data obtained from rhodamine 123 (R123). For this purpose, the rate constant for the reaction of PON with DHR123 was initially estimated. We followed the change in the rate of formation at 500 nm as a function of DHR123 concentration varying from 25 to 75 μM. **Figure 4a** shows a linear plot for the change in rate of the reaction with respect to increasing concentration of DHR123. The slope gave a bimolecular rate constant of 8200 M^{-1} s^{-1}. Using this, the bimolecular rate constant of the reaction of PON with vanillin was estimated by competition kinetics.

The reaction steps may be written as

$$\text{PON} + \text{DHR123} \xrightarrow{k_2} \text{R123} \qquad (2)$$

$$\text{PON} + \text{VAN} \xrightarrow{k_1'} \text{products} \qquad (1')$$

k_1 and k_1' represent rate constants for the same reaction but calculated by different methods.

The fraction of PON going to DHR123 in the presence of vanillin (F_0/F) is given by the equation

$$F_0/F = 1 + k_1'\,[\text{VAN}]/k_2[\text{DHR123}]$$

where F_0 is the fluorescence of rhodamine 123 in the absence of vanillin and F is the fluorescence of rhodamine 123 in the presence of vanillin. The values of F_0 and F used were from **Figure 1b**. **Figure 4b** shows a linear plot for the variation of $F_0/F - 1$ versus [VAN]/[DHR123].

The ratio k_1'/k_2 is the slope of the straight line. Using the value of k_2 (8200 M^{-1} s^{-1}), the value of k_1' was obtained as 1300 M^{-1} s^{-1}.

This value of the rate constant obtained by competitive kinetics (k_1') was comparable to the rate constant (k_1) estimated by stopped-flow measurement.

Chromatographic separation of vanillin showed a single peak with a retention time of 7.27 min (**Figure 5a**). After reaction

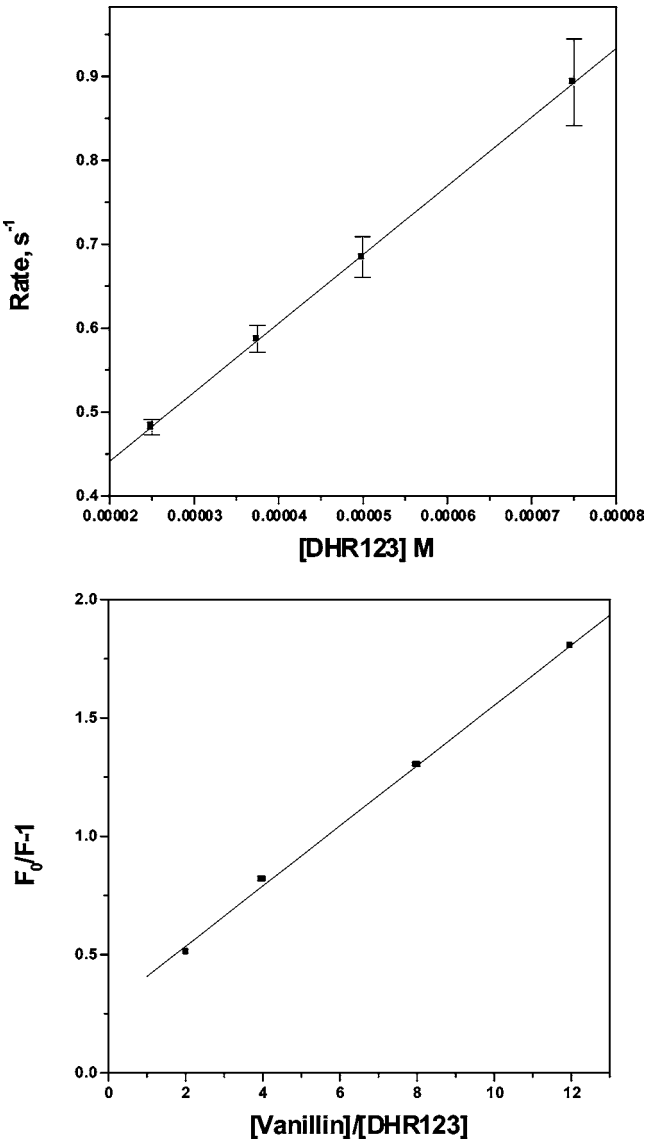


Figure 4. (**a**, top) Linear plot showing the change in rate of the reaction at 500 nm as a function of DHR123 concentration after mixing with 5 μM PON at pH 7.0. (**b**, bottom) Linear plot showing the variation of (F_0/F − 1) as a function of the ratio of concentration of vanillin to that of DHR123 to estimate the rate constant by competition kinetics. Details are explained under Results and Discussion.

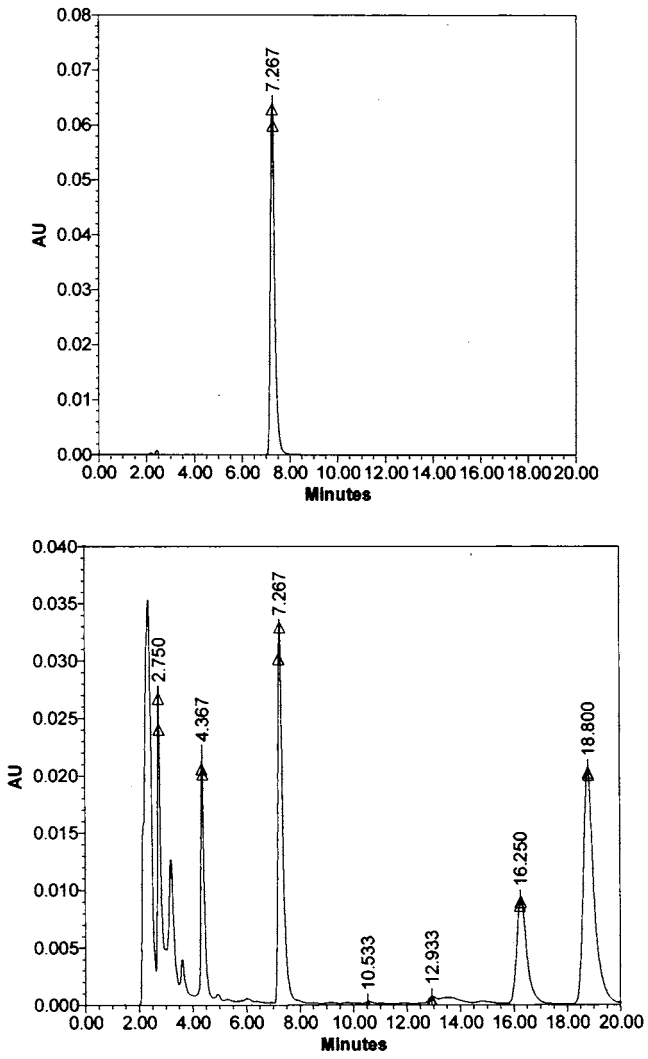


Figure 5. (**a**, top) HPLC profile of vanillin (500 μM) at pH 7.0. (**b**) Separation of different products of the reaction of vanillin with 500 μM PON at pH 7.0. Column conditions: mobile phase, 0.1 M citric acid, 15% acetonitrile, pH 2.0; flow rate, 0.5 mL/min; wavelength, 350 nm.

of PON with vanillin at pH 7, five major products were obtained as illustrated by the chromatogram shown in **Figure 5b**. Retention times of these products were 2.75, 4.37, 12.93, 16.25, and 18.80 min, respectively (**Figure 5b**). The peak corresponding to 4.37 min was identified as a hydroxylation product of vanillin, that is, 2,4-dihydroxy-3-methoxybenzaldehyde, using a commercial standard (**Figure 5b**). The peaks at 2.37, 3.20, and 3.61 min correspond to nitrate, nitrite, and azide, respectively, which were confirmed by injecting the appropriate standards. The other major products could be quinone and hydroxylated, nitration, and nitrosylation products of vanillin. HPLC analysis of the reaction of PON with phenols was reported earlier (*41*), in which a number of products have been characterized. The reaction was also monitored at 265, 285, and 425 nm, but no new products other than those observed at 350 nm were found. Only the change in the relative absorption intensity varied.

Pulse radiolysis studies of vanillin with various radicals have been reported by Mahal and co-workers (*42*). For the sake of comparison, reactions of vanillin with hydroxyl radicals and nitrogen dioxide radicals generated by pulse radiolysis were carried out (**Figure 6**). Reaction of hydroxyl radicals with vanillin produced transients absorbing at 410 nm, which have been attributed to phenoxyl radicals (*42*). The radicals decayed by second-order kinetics with a $2k/\epsilon l$ of 1.2×10^6 s^{-1}, probably due to radical−radical reaction (right inset of **Figure 6**).

In addition to hydroxyl radicals, the reaction of PON proceeds through the intermediacy of $NO_2^{\bullet}$. Therefore, the reaction of $NO_2^{\bullet}$ with vanillin was independently studied. At pH 7, vanillin (200 μM) reacted with $NO_2^{\bullet}$ radicals, producing a transient showing absorption in the 400−500 nm region with λ_{max} at 400−440 nm (**Figure 6**), and the bimolecular rate constant for the reaction was 4.2×10^6 M^{-1} s^{-1}. The reaction of $NO_2^{\bullet}$ with vanillin at the same pH gave a similar transient with reduced absorbance at 410 nm with an additional peak at 320−330 nm, which might be due to adduct formation. The transient produced by $NO_2^{\bullet}$ reaction did not show any appreciable decay in 5 s (left inset of **Figure 6**). The rate constant for $NO_2^{\bullet}$ reaction with vanillin was much less than that of the $^{\bullet}OH$ radical. This suggested that although the spectra appeared to be very similar, they might correspond to different transients probably due to the formation of radical adducts that might finally lead to the

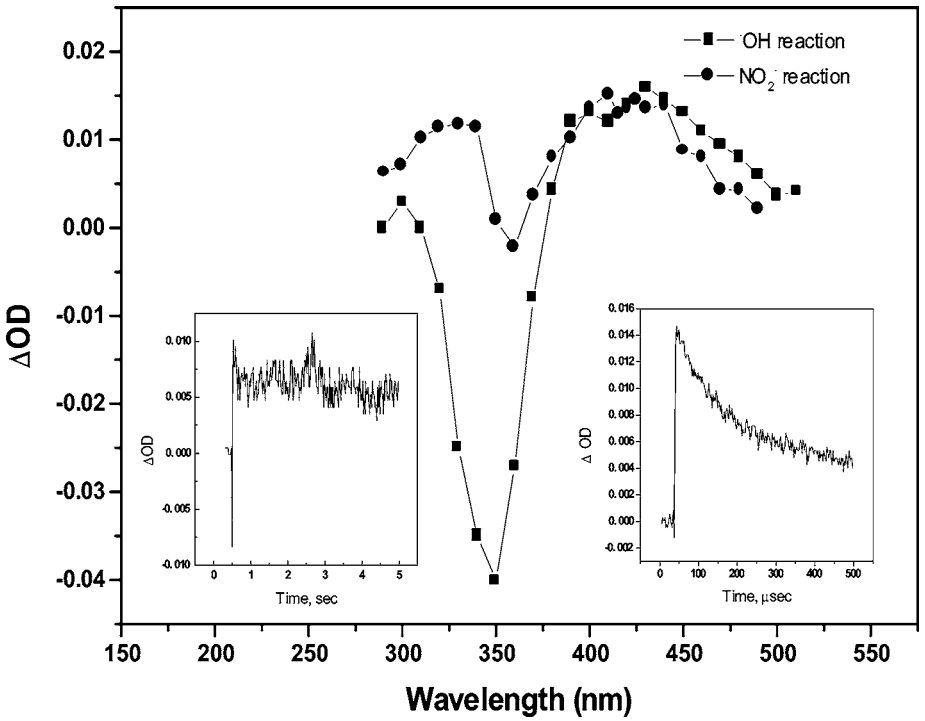


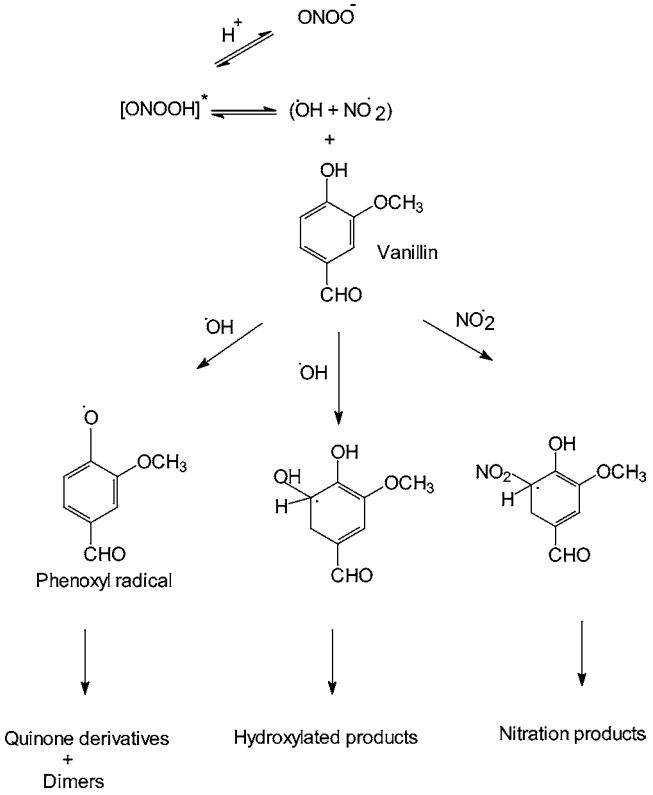
Figure 6. (■) Difference absorption spectrum of the transient obtained 4.5 μs after pulse radiolysis of N_2O-saturated aqueous solution containing 1×10^{-4} M vanillin at pH 7.0. (Right inset) Absorption–time plot showing the decay of the transient at 430 nm. (●) Difference absorption spectrum of the transient obtained 1.8 ms after pulse radiolysis of N_2O-saturated aqueous solution containing 200 μM vanillin and 0.02 M sodium nitrite at pH 7.0. (Left inset) Transient formed during the reaction of $NO_2^{\bullet}$ with vanillin showed no decay in 5 s time scale.

formation of nitrated products. The electron-transfer reaction between $NO_2^{\bullet}$ and vanillin is a thermodynamically unfavorable process, due to the fact that the reduction potential of the couple ($NO_2^{\bullet}/NO_2^-$) is 1 V versus NHE, whereas that for vanillin$^{\bullet+}$/vanillin is 0.862 V versus NHE (*23*). Hence, the major reaction pathway may be the radical adduct formation by the addition of $NO_2^{\bullet}$ radical to the ortho position of phenolic OH as indicated in **Scheme 1**.

PON has a pK_a of 6.8 at 25 °C and, therefore, partially undergoes protonation at physiological pH (*1*). The protonated form, peroxynitrous acid (ONOOH), is very unstable and decomposes to intermediates formed with the reactivity of $^{\bullet}$OH and $NO_2^{\bullet}$ (*2*). PON can cause both one- and two-electron oxidations of the substrates (*2*). The direct oxidation is mediated by an oxygen atom transfer process. Among the various substrates of interaction with PON, phenols are well studied (*41*). The possible reaction mechanisms for the actual reaction involving vanillin and PON are outlined in **Scheme 1**. The reaction of PON with vanillin can proceed by hydroxyl-type radical reactivity, producing hydroxylated adducts (**Scheme 1**). The hydroxyl radical adducts may lose a proton to give hydroxylated vanillin. PON also generates the $NO_2^{\bullet}$ radical, which may add to vanillin, giving radical adducts, which may lose a proton to yield the nitrated products (**Scheme 1**). The $NO_2^{\bullet}$ reaction is much slower as compared to the $^{\bullet}$OH-like radical reaction and may be possible only at very high concentrations of vanillin. Although thermodynamically not a favorable process, $NO_2^{\bullet}$ may also oxidize vanillin to give same phenoxyl radicals.

Because vanillin is used for direct human consumption and PON is generated inside cells, these observations, if extended to in vivo systems, may have functional significance. However, dietary compounds may undergo structural changes or degradation during metabolism. These processes are important in determining their role in disease conditions and also for understanding their mechanism of action. The "bioavailability" of exogenous antioxidants depends on their absorption, biodistribution, and bioactivity after reaching the site of action. Some of these dietary antioxidants may be modified by xenobiotic metabolizing enzymes such as cytochrome P450 dependent

Scheme 1. Possible Mechanism and Products Formed during the Reaction of PON with Vanillin



monooxygenases, conjugating enzymes of phase II biotransformation (UDP glucuronosyl transferases or phenol sulfotransferases), esterases, β-glucosidases, or some very specific enzymes. The metabolic products may retain or lose their antioxidant activity in the body. Carotenoids are cleaved to apocarotenoids (*43*), whereas α-tocopherol is oxidized to α-tocopherol hydroquinones (*44*) and polyphenols are conjugated to glucuronic acid and/or sulfate in the liver (*45*). The blocking of hydroxyl groups on the parent molecule due to conjugation results in loss of antioxidant activity of polyphenols, whereas α-tocopherol hydroquinones retain their antioxidant activity in the body (*46*). Antioxidants such as glutathione and vitamin C are regenerated enzymatically from their metabolites (*47*).

In conclusion, our studies show that vanillin inhibits PON-induced nitration and oxidation reactions. With the help of a stopped-flow technique, pulse radiolysis, and HPLC analysis, the kinetics of the reaction was studied and some of the products have been identified. Some of the important mechanistic steps involved during the progress of the reaction are proposed.

ABBREVIATIONS USED

PON, peroxynitrite; ROS, reactive oxygen species; HPLC, high-performance liquid chromatography; $NO_2^{\bullet}$, nitrogen dioxide; $^{\bullet}OH$, hydroxyl radical; $NO^{\bullet}$, nitric oxide; $N_3^{\bullet}$, azide radical; H_2O_2, hydrogen peroxide; DPPH, diphenylpicrylhydrazyl radical.

ACKNOWLEDGMENT

We acknowledge the encouragement and support of Dr. T. Mukherjee and Dr. Hari Mohan of the Radiation Chemistry and Chemical Dynamics Division, BARC.

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Received for review April 28, 2003. Revised manuscript received October 23, 2003. Accepted October 24, 2003.

JF030319D