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Quercetin-Dependent Inhibition of Nitration Induced by Peroxidase/H_2O_2/Nitrite Systems in Human Saliva and Characterization of an Oxidation Product of Quercetin Formed during the Inhibition

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Local pH in the oral cavity can decrease to below 7 at the site where acid-producing bacteria are proliferating. Effects of pH on nitration of 4-hydroxyphenylacetic acid were studied using dialyzed human saliva. Dialyzed saliva nitrated 4-hydroxyphenylacetic acid to 4-hydroxy-3-nitrophenylacetic acid in the presence of nitrite and H_2O_2. The rate of the nitration was dependent on pH, and the maximal rate was observed between pH 5.5 and 7.2. The optimum pH seemed to reflect rates of formation of nitrogen dioxide and 4-hydroxyphenylacetic acid radicals. Quercetin inhibited the nitration. The quercetin-dependent inhibition might be due to scavenging of nitrogen dioxide and 4-hydroxyphenylacetic acid radicals, which were formed by salivary peroxidase-dependent oxidation of nitrite and 4-hydroxyphenylacetic acid, respectively, and competition with nitrite and 4-hydroxyphenylacetic acid for peroxidase in saliva. An oxidation product of quercetin was formed during inhibition of the nitration by quercetin. The oxidation product was identified as 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone. This component could also be oxidized by salivary peroxidase and nitrogen dioxide radicals. The oxidation products were 2,4,6-trihydroxyphenylglyoxylic and 3,4-dihydroxybenzoic acids. On the basis of the results, the significance of quercetin for inhibition of nitrogen dioxide formation and for scavenging of nitrogen dioxide radicals in the oral cavity is discussed.

KEYWORDS: Inhibition of nitration; nitration; nitrite; oxidation product of quercetin; quercetin; salivary peroxidase

INTRODUCTION

Nitrate is secreted into the oral cavity as a salivary component. Bacteria in the cavity can reduce nitrate to nitrite (*1*, *2*), and the concentration of nitrite in mixed whole saliva ranges from 0.05 to 0.3 mM (*3*). It has been reported that the concentration of nitrite increases after the ingestion of nitrate-rich foods (*4*, *5*). On the other hand, vegetables and fruits contain quercetin glycosides. When foods that contain quercetin glycosides are ingested, the glycosides are hydrolyzed to quercetin, and the quercetin can be oxidized by peroxidase-dependent reactions in the oral cavity (*6*). Salivary peroxidase secreted from salivary glands and myeloperoxidase derived from polymorphonuclear leukocytes are found as peroxidases in the cavity (*7*, *8*). Hydrogen peroxide, a substrate of salivary peroxidase and myeloperoxidase, is generated by some species of bacteria (*7*) and polymorphonuclear leukocytes (*9*−*11*). It has been reported that peroxidases can oxidize nitrite to nitrogen dioxide radicals, which can participate in the nitration of tyrosine and tyrosine residues in proteins (*12*−*17*), by the following reactions:

$$\text{peroxidase} + H_2O_2 \rightarrow \text{compound } \mathbf{I} + H_2O \quad (1)$$

$$\text{compound } \mathbf{I} + NO_2^- \rightarrow \text{compound } \mathbf{II} + NO_2 \quad (2)$$

$$\text{compound } \mathbf{II} + NO_2^- + 2H^+ \rightarrow \text{peroxidase} + NO_2 + H_2O \quad (3)$$

Here, compound **I** is a complex of peroxidase and atomic oxygen and compound **II** is the one-electron reductant of compound **I**. We have suggested that myeloperoxidase from salivary leukocytes and salivary peroxidase can also oxidize nitrite to nitrogen dioxide radicals (*18*, *19*). The nitration induced by nitrogen dioxide radicals can be inhibited by polyphenols (*18*). Polyphenols can also inhibit the nitration induced by nitrous acid and peroxynitrite (*20*, *21*).

During polyphenol-dependent inhibition of nitration, polyphenols including quercetin are oxidized (*18*, *21*). It has been

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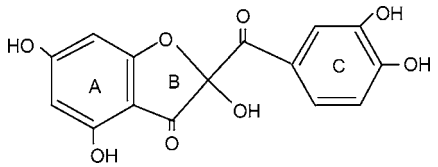

Figure 1. Structure of compound **1**.

reported that a stable oxidation product of quercetin can be separated by HPLC and that the product gives 2,4,6-trihydroxyphenylglyoxylic acid and 3,4-dihydroxybenzoic acid when oxidized by a peroxidase/H_2O_2 system (*22*). At that time, chemical characteristics of the oxidation product remain to be elucidated. Recently, an oxidation product of quercetin has been isolated from dried brown scales of onion bulbs and identified as 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone (**1**) (**Figure 1**) (*23*). This compound has also been obtained when quercetin is oxidized by metallic ions and electrolysis (*24*, *25*).

The present investigation deals with quercetin-dependent inhibition of nitration of 4-hydroxyphenylacetic acid, which is induced by peroxidase/nitrite/H_2O_2 systems in saliva, at various pH values. The reason that the effects of pH were studied was that salivary pH is usually between 7 and 8, but the local pH in the oral cavity should decrease to below 7 at sites where acid-producing bacteria are proliferating. The present investigation also deals with characterization of an oxidation product of quercetin, which was detected during quercetin-dependent inhibition of nitration of 4-hydroxyphenylacetic acid. Possible mechanisms of quercetin-dependent inhibition of nitration of 4-hydroxyphenylacetic acid and some chemical and biochemical characteristics of the oxidation product of quercetin are presented.

MATERIALS AND METHODS

Reagents. Quercetin, 4-hydroxyphenylacetic acid, 4-hydroxy-3-nitrophenylacetic acid, 3,4-dihydroxybenzoic acid, and Griess−Romijn nitrite reagents were obtained from Wako Pure Chemicals (Osaka, Japan). Horseradish peroxidase (type II) was from Sigma Japan (Tokyo). 2,4,6-Trihydroxyphenylglyoxylic acid was synthesized according to the method reported by Hargreaves et al. (*26*). Compound **1** was isolated from dried brown scales of onion bulbs as follows. Dried brown scales of onion bulbs (100 g) were successively extracted three times with hot methanol (1 L) for 2 h. After the solvent was removed, the methanol extract (10.3 g) was dissolved in water and applied to Amberlite XAD-2 resin column (20 cm × 6 cm i.d.). The resin was washed with distilled water (2 L) to eliminate water-soluble compounds, and the retained material was eluted with methanol (2 L). The methanol eluate (5.10 g) was subjected to a silica gel column. The column was sequentially eluted by increasing methanol concentration in mixtures of chloroform and methanol. The eluate of chloroform/methanol (85:15, v/v) contained compound **1**, and this fraction was further purified by HPLC. HPLC was done with a reverse phase 250 mm × 10 mm i.d. Inertsil PREP-ODS column (GL Sciences, Tokyo, Japan) developed with acetonitrile/water (30:70, v/v; containing 0.1% acetic acid) at 5.0 mL/min, and the eluate was monitored at 280 nm. Compound **1** was obtained as a brown-red amorphous solid (yield, 18.5 mg). The compound had the following characteristics: UV (methanol) λ max (log ϵ) 291 (4.39); ESI-MS (positive mode), *m*/*z* 341 ($[M + Na]^+$, 100%), 319 ($[M + H]^+$, 10%), and 301 ($[M - OH]^+$, 15%); ESI-MS (negative mode), *m*/*z* 317 ($[M - H]^-$, 100%); ^{1}H NMR (CD_3OD) δ 5.97 (s, 1H, H-6), 6.00 (s, 1H, H-8), 6.79 (d, J = 8.0 Hz, 1H, H-5′), 7.59 (dd, J = 2.3, 8.0 Hz, 1H, H-6′), and 7.62 (d, J = 2.3 Hz, 1H, H-2′); ^{13}C NMR (CD_3OD) δ 92.1 (C-8), 97.9 (C-6), 102.0 (C-4a), 105.6 (C-3), 115.6 (C-5′), 118.2 (C-2′), 125.4 (C-6′), 126.6 (C-1′), 146.1 (C-3′), 152.9 (C-4′), 160.4 (C-8a), 171.2 (C-5), 174.0 (C-7), 191.7 (C-4), and 193.4 (C-2).

Preparation of Saliva. Mixed whole saliva (∼10 mL) (pH 7.2−7.8) was collected from healthy volunteers who chewed Parafilm after their informed consent had been obtained. The saliva collected was centrifuged at 15000*g* for 5 min, and the supernatant (2 mL each) was dialyzed for 12−24 h at 4 °C against 500 mL of 10 mM sodium phosphate (pH 4, 5, 6, 7, and 8). After dialysis, saliva was centrifuged at 15000*g* for 5 min, and the supernatant was used as dialyzed saliva. Final pH values of dialyzed saliva were 4.6 ± 0.2, 5.5 ± 0.2, 6.3 ± 0.1, 7.2 ± 0.1, and 7.9 ± 0.1 (mean ± SD).

Nitration of 4-Hydroxyphenylacetic Acid. Dialyzed saliva was used to study the nitration of 4-hydroxyphenylacetic acid. The reaction mixture (1 mL) contained 0.1 mM 4-hydroxyphenylacetic acid, 1 mM $NaNO_2$, and 0.5 mM H_2O_2 in 1 mL of dialyzed saliva at pH 4.6−7.9. Reactions were started by adding H_2O_2. When required, quercetin was added to the above reaction mixture. After incubation for 0.5 min at 35 °C, the mixture was extracted with 5 mL of ethyl acetate. Prior to the extraction, the pH of the reaction mixture was adjusted to 3 by 1 M HCl. The reason that dialyzed saliva was incubated for 0.5 min was that decomposition of 0.5 mM H_2O_2 completed within 1.5 min in the presence of 1 mM $NaNO_2$ (*19*). The ethyl acetate extract was dried with anhydrous sodium sulfate, and the solvent was removed with a rotary evaporator. The residue was dissolved in 1 mL of a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v) and applied to an HPLC column to quantify 4-hydroxyphenylacetic acid and 4-hydroxy-3-nitrophenylacetic acid. Nitration of 4-hydroxyphenylacetic acid was also measured without the addition of H_2O_2 because 4-hydroxyphenylacetic acid could be nitrated by nitrite under acidic conditions. The results are presented as differences between the presence and absence of H_2O_2 in the reaction mixture.

Oxidation of Nitrite. Oxidation of nitrite was measured in the reaction mixture that was used for nitration of 4-hydroxyphenylacetic acid. Reactions were started by adding H_2O_2. After incubation for 0.5 min at 35 °C, 0.05 mL of the mixture was withdrawn and the concentration of nitrite was measured as follows. In short, the reaction mixture contained 0.05 mL of sample, 0.1 mL of 1% Griess−Romijn reagent, and 0.85 mL of 50 mM KH_2PO_4/KCl/HCl (pH 2.0). The concentration of nitrite was determined from the absorption at 540 nm after 15 min of incubation at 35 °C.

Oxidation of Quercetin. Quercetin was oxidized in the reaction mixture (1 mL) that contained 0.1 mM 4-hydroxyphenylacetic acid, 5−30 μM quercetin, and 0.5 mM H_2O_2 in a mixture of 0.1 mL of dialyzed saliva and 0.9 mL of 50 mM sodium phosphate (pH 4.5−7.8). When required, 1 mM $NaNO_2$ was added to the above reaction mixture. After incubation for defined periods, 10 μL of the reaction mixture was injected into an HPLC column to quantify quercetin and the oxidation product. To prepare the oxidation product of quercetin for LC-MS analysis, quercetin was oxidized in the reaction mixture that contained 50 μM quercetin, 0.1 mM HPA, 1 mM $NaNO_2$, and 0.5 mM H_2O_2 in 1 mL of dialyzed saliva at pH 7.8. After incubation for 0.5 min at 35 °C, the pH of the mixture was adjusted to 3 by 1 M HCl. The acidified mixture was extracted twice with 5 mL of ethyl acetate. The ethyl acetate extracts were combined and dried with anhydrous sodium sulfate, and the solvent was removed with a rotary evaporator. The residue was dissolved in 1 mL of a mixture of acetonitrile and 5 mM ammonium acetate (1:5, v/v) and applied to an LC-MS system.

Characterization of Compound 1. Compound **1** (50 μM) was dissolved in 50 mM sodium phosphate (pH 4.5−8.5), and the absorption spectra were measured using a UV-260 double-beam spectrophotometer (Shimadzu, Kyoto, Japan). Peroxidase-dependent oxidation of compound **1** was also measured using the spectrophotometer. The reaction mixture (1 mL) contained 50 μM compound **1**, 0.1−0.5 mM H_2O_2, and 0.2 μg/mL of horseradish peroxidase or 0.1 mL of dialyzed saliva in 1 or 0.9 mL of 50 mM sodium phosphate (pH 4.5−7.2), respectively. The oxidation products of compound **1**, which were formed by horseradish peroxidase/H_2O_2 and salivary peroxidase/H_2O_2 systems, were analyzed by HPLC after incubation for 5 min.

HPLC. HPLC was performed using a reverse phase 150 mm × 6 mm i.d. Shim-pack CLC ODS column (Shimadzu, Kyoto, Japan). Components separated by the column were detected with an SPD-M10A spectrophotometric detector with a photodiode array (Shimadzu). The mobile phase used for the separation of 4-hydroxyphenylacetic acid and 4-hydroxy-3-nitrophenylacetic acid was a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v). Oxidation of quercetin was measured using

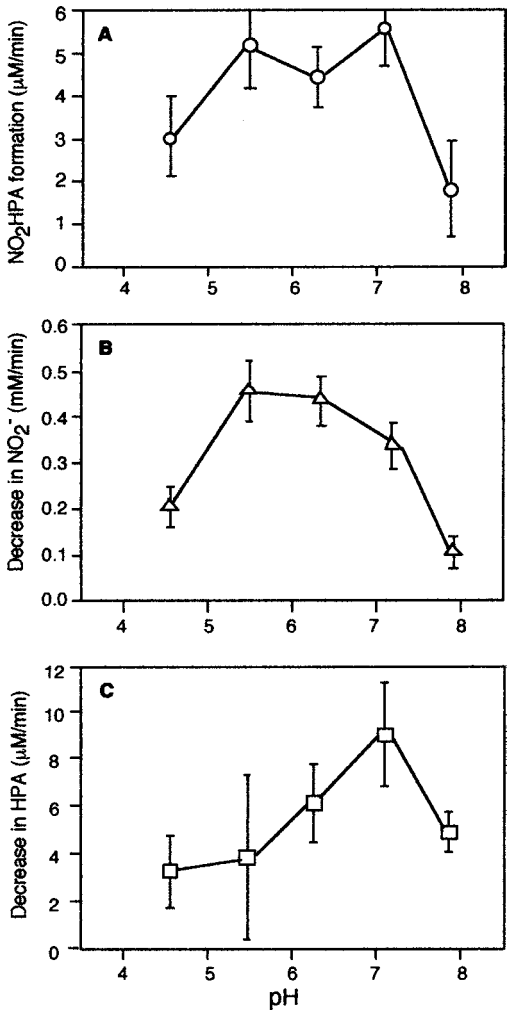

Figure 2. Formation of 4-hydroxy-3-nitrophenylacetic acid (**A**) and decrease in concentrations of nitrite (**B**) and 4-hydroxyphenylacetic acid (**C**) by peroxidase in dialyzed saliva. Data plots represent averages of four experiments (bars, SD). HPA, 4-hydroxyphenylacetic acid; NO_2HPA, 4-hydroxy-3-nitrophenylacetic acid.

a mixture of methanol and 25 mM KH_2PO_4 (3:2, v/v) as mobile phase. An oxidation product of quercetin was separated using mixtures of methanol and 25 mM KH_2PO_4 (1:2, v/v; pH 4.5) for quantification of the oxidation product and of acetonitrile and 5 mM ammonium acetate (1:5, v/v; pH 7.2) for measurement of mass spectra using an 1100 LC-MSD SL spectrometer (Agilent Technologies) as mobile phases. The components separated by the above HPLC systems were quantified from peak areas on chromatograms at 280 nm for 4-hydroxyphenylacetic acid and compound **1** and at 360 nm for 4-hydroxy-3-nitrophenylacetic acid and quercetin. Oxidation products of compound **1** were separated using a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v) as mobile phase, and components separated were detected at 280 nm. The flow rate of the above mobile phases was 1 mL/min.

Oxygen Evolution. Salivary peroxidase-dependent oxygen evolution was measured using an oxygen electrode (Rank Brothers, Cambridge, U.K.) in the reaction mixture that contained 0.5 mM H_2O_2 in a mixture of 0.2 mL of dialyzed saliva and 0.8 mL of 50 mM sodium phosphate (pH 4.5−7.8) at 35 °C.

RESULTS AND DISCUSSION

Nitration of 4-Hydroxyphenylacetic Acid. It has been reported that 4-hydroxyphenylacetic acid, tyrosine, and tyrosine residues in proteins are nitrated by peroxidase/nitrite/H_2O_2 systems. **Figure 2** shows the effects of pH on the nitration of 4-hydroxyphenylacetic acid. The phenolic acid was nitrated to

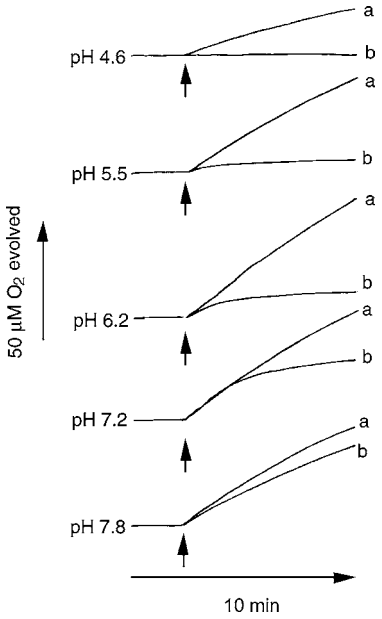

Figure 3. Inhibition of oxygen evolution by nitrite. Where indicated by arrows, 0.5 mM H_2O_2 was added: a, no addition; b, 1 mM $NaNO_2$.

4-hydroxy-3-nitrophenylacetic acid when nitrite and H_2O_2 were added to dialyzed saliva. The nitrated component was separated by HPLC. The retention time (7.6 min) and the absorption spectrum (λ_{max}; 215, 275, and 357 nm) in the mobile phase were identical with those of authentic 4-hydroxy-3-nitrophenylacetic acid. The rate of nitration was dependent on pH, and a broad peak was observed between pH 5.5 and 7.2 (**Figure 2A**). Essentially the same result was obtained when the reaction mixture for nitration was directly analyzed by HPLC. **Figure 2B** shows the effects of pH on the consumption of nitrite by dialyzed saliva during nitration. Nitrite was consumed at all pH values examined, and the maximal rate of the consumption was observed between pH 5.5 and 6.2. This result suggests that the oxidation of nitrite was faster under weak acidic conditions. It has been reported that rate constants for the reaction between nitrite and myeloperoxidase in the presence of H_2O_2 are larger under acidic conditions than under neutral and alkaline conditions (*27*). As salivary peroxidase catalyzes the following reactions evolving molecular oxygen (*28*),

$$\text{salivary peroxidase} + H_2O_2 \rightarrow \text{compound } \mathbf{I} + H_2O \quad (4)$$

$$\text{compound } \mathbf{I} + H_2O_2 \rightarrow \text{salivary peroxidase} + O_2 + H_2O \quad (5)$$

we examined the effects of nitrite on the oxygen evolution (**Figure 3**). The oxygen evolution was inhibited by nitrite, and the inhibitory effect became larger as the pH was decreased from 7.8 to 4.5. This result suggests that nitrite was oxidized by competing with H_2O_2 for compound **I** of peroxidase in saliva and by donating electrons to compound **II** formed from compound **I** (see reactions 1−3).

4-Hydroxyphenylacetic acid was also consumed during the nitration, and the maximal rate of consumption was observed at pH ∼7.2 (**Figure 2C**). It has been discussed that nitration of 4-hydroxyphenylacetic acid by peroxidase/nitrite/H_2O_2 systems is due to a reaction between nitrogen dioxide and 4-hydroxyphenylacetic acid radicals (*16*). By comparing the effects of pH on nitration, nitrite oxidation, and consumption of 4-hydroxyphenylacetic acid, we may be able to deduce that the nitration in dialyzed saliva is controlled by oxidation rates of nitrite and

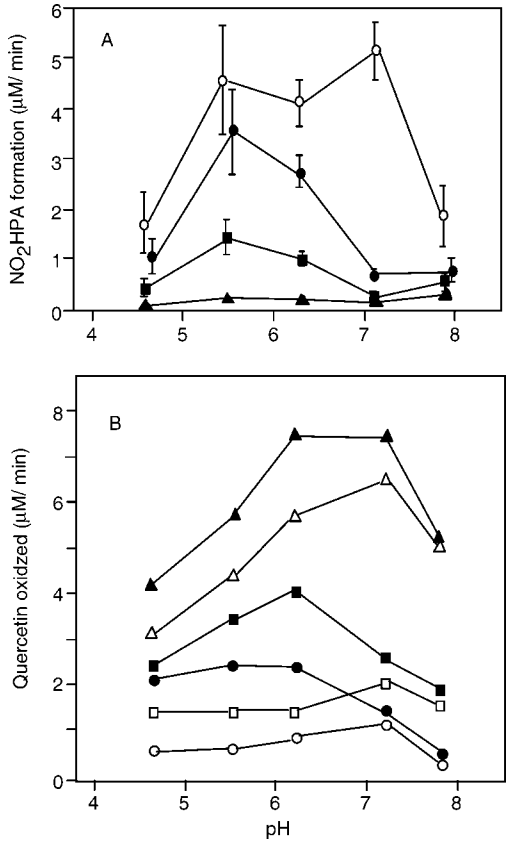

Figure 4. Quercetin-dependent inhibition of formation of 4-hydroxy-3-nitrophenylacetic acid and oxidation of quercetin: (**A**) inhibition of nitration [○, without quercetin; ●, 5 μM quercetin; ■, 10 μM quercetin; ▲, 30 μM quercetin; data plots represent averages of three experiments (bars, SD)]; (**B**) enhancement of oxidation of quercetin by nitrite (○, 5 μM quercetin; ●, 5 μM quercetin + 1 mM $NaNO_2$; □, 10 μM quercetin; ■, 10 μM quercetin + 1 mM $NaNO_2$; △, 30 μM quercetin; ▲, 30 μM quercetin + 1 mM $NaNO_2$). The rate of oxidation of quercetin was calculated using data after 2 min of incubation. Each data plot represents the average of two experiments. NO_2HPA, 4-hydroxy-3-nitrophenylacetic acid.

the phenylacetic acid to their radicals. Rates of consumption of 4-hydroxyphenylacetic acid were comparable to rates of the formation of 4-hydroxy-3-nitrophenylacetic acid but much lower than those of oxidative consumption of nitrite at all pH values examined. This result suggests that 4-hydroxyphenylacetic acid was mainly consumed by nitration, especially under acidic conditions. Nitrogen dioxide radicals formed might be transformed to dinitrogen tetraoxide to react with water.

Inhibition of Nitration by Quercetin. Quercetin inhibited the nitration, and the inhibition by low concentrations of quercetin were larger at neutral and alkaline pH than at acidic pH (**Figure 4A**). Quercetin (5 μM) inhibited the nitration by ~80% at pH 7.2, whereas that concentration of quercetin inhibited the nitration by ~25% at pH 5.5. A high concentration of quercetin (30 μM) significantly inhibited the nitration at all pH values examined. **Figure 4B** shows the effect of nitrite on the oxidation of quercetin at various pH values. Quercetin was oxidized in the absence of nitrite, and the optimum pH for the oxidation was ~7.2. The oxidation was enhanced by nitrite. The degree of the enhancement by nitrite was dependent on pH and the concentration of quercetin. Broadly speaking, the enhancement was larger at low pH values and in the presence of low concentrations of quercetin.

Identification of Oxidation Product of Quercetin. During the oxidation of quercetin, its oxidation products were formed,

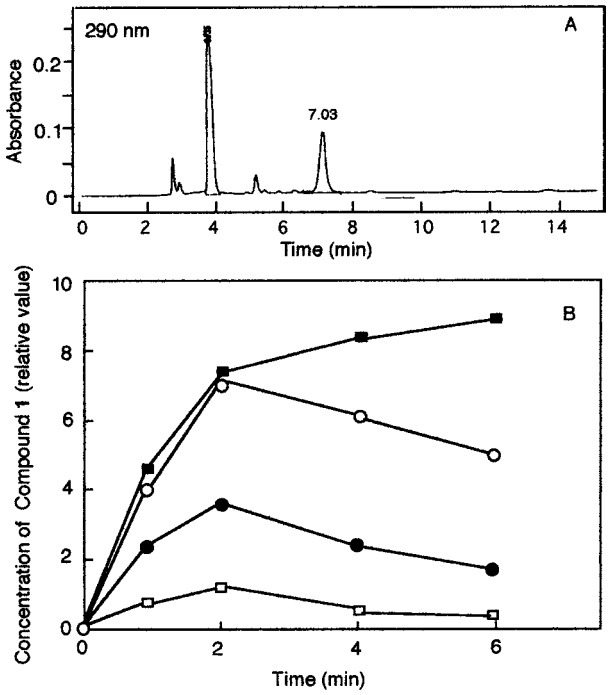

Figure 5. Separation of an oxidation product of quercetin by HPLC and its changes in concentration: (**A**) HPLC profile of oxidation product of quercetin (components with retention times of 3.8 and 7.03 min are 4-hydroxyphenylacetic acid and compound **1**, respectively); (**B**) time courses of changes in concentration of compound **1** (initial concentration of quercetin was 5 μM; ○, pH 4.6; ●, pH 5.5; □, pH 6.2; ■, pH 7.2).

and the main product could be separated by HPLC (**Figure 5A**). Its retention time was 7.03 min in the mobile phase of acetonitrile and 5 mM ammonium acetate (1:5, v/v; pH 7.2). When mixtures of methanol and 25 mM KH_2PO_4 (1:2 and 1:5, v/v; pH 4.5) were used as mobile phase, the retention times were 7.15 and 15.5 min, respectively. The absorption spectrum of the oxidation product had peaks at 296 and 322 nm in the mobile phase at pH 7.2 and at 294 and 322 (shoulder) nm in the mobile phases at pH 4.5. Its mass spectrum was measured using an LC-MS apparatus with a mixture of acetonitrile and 5 mM ammonium acetate (1:5, v/v) as mobile phase. The negative electrospray ionization (ESI) MS showed the $[M - H]^-$ ion at *m*/*z* 317. The data of retention times, UV spectra, and ESI-MS were identical with those of compound **1** (molecular weight, 318), which was isolated from dried brown scales of onion bulbs, indicating that the oxidation product of quercetin detected in this study was compound **1**. It has been reported that compound **1** formed by cation-induced or electrochemical oxidation of quercetin also shows the $[M - H]^-$ ion at *m*/*z* 317 (*29*).

Figure 5B shows time courses of changes in concentration of compound **1**, which was formed when quercetin was oxidized by dialyzed saliva in the presence of both nitrite and H_2O_2. Its formation was slowest at pH 6.3. At acidic pH values, concentrations of compound **1** decreased after attaining maximal values, but at pH 7.2, the concentration increased during the incubation period. This result suggests that compound **1** formed by the oxidation of quercetin could be oxidized further by peroxidase/nitrite/H_2O_2 systems in dialyzed saliva.

Characteristics of Compound 1. The absorption spectrum of the oxidation product of quercetin (compound **1**) was affected by pH, and the product seemed to be oxidized further by peroxidase in dialyzed saliva (**Figure 5B**). We studied some

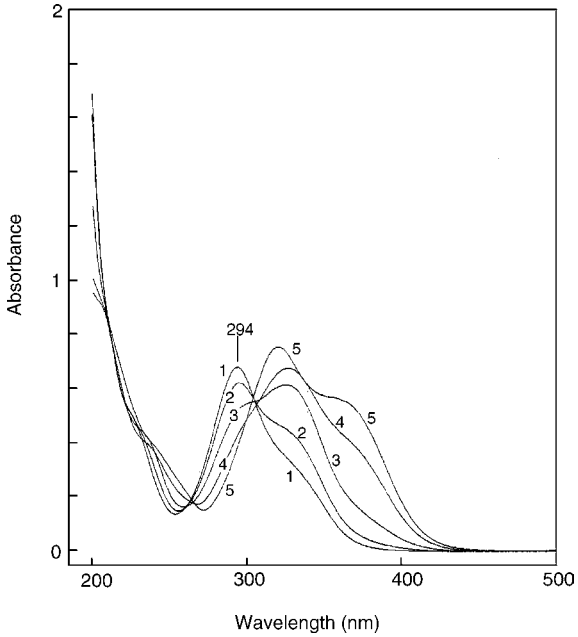

Figure 6. Effects of pH on absorption spectrum of compound **1**: trace 1, pH 4.5; trace 2, pH 5.5; trace 3, pH 6.5; trace 4, pH 7.5; trace 5, pH 8.5.

chemical and biochemical characteristics of isolated compound **1**. **Figure 6** shows absorption spectra of isolated compound **1** at various pH values. At acidic pH, compound **1** had an absorption peak at 294 nm with a shoulder at about 320 nm. The peak at 294 nm decreased and the absorption band around 320 nm increased as the pH was increased from 4.5 to 8.5. When the pH was higher than 7.5, an additional absorption band appeared around 370 nm. Compound **1** decomposed slowly at alkaline pH. The absorption bands around 320 and 370 nm disappeared when the pH was decreased from 8.5 to 4.5 by adding 1 M H_3PO_4. The absorption spectrum at pH 4.5 was the same as that of compound **1**, which was dissolved in 50 mM sodium phosphate (pH 4.5). The results suggest that pH-dependent changes in the absorption spectrum were due to dissociation of protons from phenolic OH groups. pK_{a1} and pK_{a2} were estimated to be about 5.7 and 7.1, respectively, from pH-dependent changes in the spectrum. At present, it is not certain which hydrogen atoms dissociate as protons, but it is likely that OH groups of ring A (see **Figure 1**) dissociate if the pK_a values of flavonoids are taken into consideration.

Panels **A** and **B** of **Figure 7** show the oxidation of compound **1** by a peroxidase/H_2O_2 system in dialyzed saliva at pH 6.2. The oxidation was observed as decreases in absorbance at about 295 and 320 nm, and the oxidation was enhanced by nitrite. Such decreases in absorbance were also observed at other pH values, and maximal enhancement by nitrite was observed at pH 6.2 (**Figure 7B**). The oxidation of compound **1** was also studied using a horseradish peroxidase/H_2O_2 system at pH 5.1 (**Figure 7C**). After the addition of H_2O_2, absorbance around 295 nm initially decreased and then increased. Absorbance around 490 nm increased after the addition of H_2O_2, producing a red component (spectra 3−5). The red component was relatively stable at pH 5.1, and the red color disappeared when kept for 24 h at 4 °C in the dark. Such a red component was also formed at other pH values. The red color disappeared within 4 h at 0 °C at pH 7.2.

Oxidation products of compound **1** formed by the above two systems were analyzed by HPLC. Three components were separated as horseradish peroxidase-dependent oxidation products of compound **1** (**Figure 8A**). A component with a retention time of 3.2 min had absorption peaks at 205 and 293 nm, and a component with a retention time of 3.7 min had absorption peaks at 207, 255, and 292 nm in the mobile phase used in this study. The former and the latter components were identified as 2,4,6-trihydroxyphenylglyoxylic and 3,4-dihydroxybenzoic acids, respectively, by comparing the retention times and absorption spectra with those of the authentic compounds. A component with a retention time of 5.3 min was a red component and had an absorption spectrum as shown in **Figure 8C** (spectrum a). When compound **1** was oxidized by dialyzed saliva under the conditions of **Figure 7A**,**B**, the oxidation products were 2,4,6-trihydroxyphenylglyoxylic and 3,4-dihydroxybenzoic acids. The production of the above two phenolics has been reported when quercetin was oxidized by peroxidases (*22*, *30*, *31*).

The red component was characterized further. Hydrogen peroxide (0.1 mM) was added to the reaction mixture in which absorption around 490 nm attained to a maximum value at pH 5.1 (**Figure 7D**, spectrum 2), but no further changes in the absorption spectrum were observed (spectrum 3). The red component was not oxidized by peroxidase in saliva, either (data not shown). By the addition of a small grain of sodium borohydride, the absorption band around 490 nm disappeared (spectrum 4). This decrease in absorbance was also observed by the addition of 0.2 mM ascorbic acid or glutathione. These results suggest that the red component might have a quinone-type structure. Ascorbic acid was added to the reaction mixture in which the red component was formed, and then the mixture was analyzed by HPLC (**Figure 8B**). In addition to 2,4,6-trihydroxyphenylglyoxylic acid (retention time of 3.2 min) and 3,4-dihydroxybenzoic acid (retention time of 3.7 min), a new component with a retention time of 7.5 min was detected. The absorption spectrum of the new component (**Figure 8C**, spectrum b) had a peak at 373 nm, suggesting that the new component was not compound **1**.

Possible Reactions Occurring during Quercetin-Dependent Inhibition of Nitration. Quercetin inhibited the nitration of 4-hydroxyphenylacetic acid, and the degree of the inhibition was dependent on pH, especially when the concentration of quercetin was low (**Figure 4A**). At alkaline pH values, 5 μM quercetin significantly inhibited the nitration of 4-hydroxyphenylacetic acid, but nitrite did not significantly inhibit initial rates of oxygen evolution (**Figure 3**) or enhance the oxidation of quercetin (**Figure 4B**). The results suggest that under alkaline conditions, quercetin might mainly inhibit the nitration by suppressing the oxidation of 4-hydroxyphenylacetic acid to the radicals by peroxidase in saliva, scavenging of 4-hydroxyphenylacetic acid radicals, or both. The quercetin-dependent inhibition of oxidation of 4-hydroxyphenylacetic acid can be deduced from the results that quercetin itself was oxidized by peroxidase in saliva in the presence of 4-hydroxyphenylacetic acid (**Figure 4B**). Quercetin-dependent reduction of 4-hydroxyphenylacetic acid radicals can be deduced from the report that diphenols can reduce radicals of monophenols (*32*).

Smaller inhibition of nitration of 4-hydroxyphenylacetic acid by 5 μM quercetin at lower pH may be due to a rapid consumption of quercetin. This is supported by the results that oxidation of 5 μM quercetin was greatly enhanced by nitrite under acidic conditions (**Figure 4B**). Indeed, no quercetin was detected after 0.5 min of incubation under the conditions of nitration at acidic pH values (data not shown). The significant enhancement of oxidation of 5 μM quercetin by nitrite at acidic pH values may be explained by rapid reaction between nitrogen dioxide radicals and quercetin.

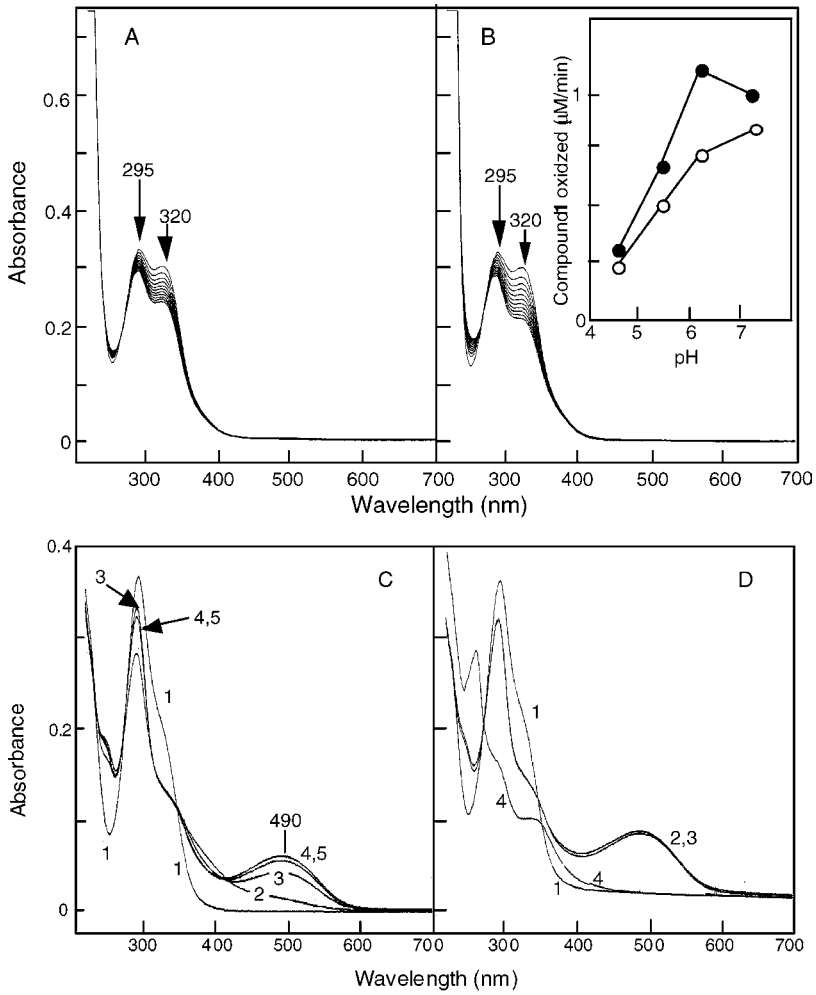

Figure 7. Peroxidase-dependent oxidation of compound **1**. (**A**, **B**) Oxidation of compound **1** by peroxidase in saliva (**A**, no addition; **B**, 1 mM $NaNO_2$). Immediately after the addition of 0.5 mM H_2O_2, scanning was repeated every 1.67 min. [**B** (inset)] Oxidation rates of compound **1**: ○, no addition; ●, 1 mM $NaNO_2$. (**C**) Horseradish peroxidase-dependent oxidation of compound **1**: trace 1, no addition; trace 2, immediately after the addition of 0.1 mM H_2O_2; trace 3, 1.67 min after the addition of H_2O_2; trace 4, 3.34 min after the addition of H_2O_2; trace 5, 5.01 min after the addition of H_2O_2. (**D**) Reduction of a product formed by horseradish peroxidase-dependent oxidation of compound **1**: trace 1, no addition; trace 2, 10 min after the addition of 0.1 mM H_2O_2; trace 3, 5 min after the further addition of 0.1 mM H_2O_2 to trace 2; trace 4, addition of one grain of sodium borohydride to trace 3.

Nitrite-dependent enhancement of oxidation of 30 μM quercetin was small (**Figure 4B**). This result suggests that, at high concentrations of quercetin, the flavonol was oxidized by both peroxidase itself in saliva and nitrogen dioxide radicals generated by peroxidase-catalyzed reactions. According to this, significant inhibition of nitration of 4-hydroxyphenylacetic acid by 30 μM quercetin may be explained by quercetin-dependent inhibition of the oxidation of nitrite and scavenging of nitrogen dioxide radicals. In addition, quercetin-dependent inhibition of oxidation of 4-hydroxyphenylacetic acid and scavenging of its radicals may also contribute to the inhibition of nitration. We have discussed above the conclusion that quercetin may mainly inhibit the nitration by scavenging nitrogen dioxide radicals as reactive nitrogen species, but participation of other reactive nitrogen species such as peroxynitrite in the nitration under acidic conditions cannot be excluded. This is deduced by the fact that peroxynitrite can contribute to nitration and peroxynitrite can be formed by the reaction between nitrite and H_2O_2 under acidic conditions (*12*, *20*).

Formation of Compound 1 and Its Reactions. During the inhibition of nitration by quercetin, the flavonol was oxidized, producing compound **1**, and the concentration of compound **1** formed decreased gradually under acidic conditions (**Figure 5C**). Compound **1** was oxidized by peroxidase in saliva, and the oxidation was enhanced by nitrite (**Figure 7B**). These results suggest that compound **1** as well as quercetin could scavenge nitrogen dioxide radicals by their antioxidative activities. Compound **1** could also inhibit lipid peroxidation (unpublished result). The rate of formation of compound **1** was minimal at pH 6.2 in the presence of nitrite (**Figure 5C**). As maximal enhancement of oxidation of compound **1** by nitrite was observed at pH 6.2 (**Figure 7B)**, this result may be explained by the rapid oxidation of compound **1** formed from quercetin.

Formation of a compound similar to compound **1** has been reported when quercetin was oxidized by peroxidases (*22*, *31*, *33*), nitrous acid (*34*, *35*), and polyphenol oxidase (*36*). During the oxidation of compound **1** by peroxidase/H_2O_2 systems in saliva and horseradish peroxidase/H_2O_2 systems, 2,4,6-trihydroxyphenylglyoxylic and 3,4-dihydroxybenzoic acids were formed. It has been reported that the above two phenolic acids are also formed by peroxidase-dependent oxidation of a compound **1**-like component and that the two phenolic acids are present in the dried brown scales of onion bulbs (*22*). It has also been reported that a red component with an absorption peak around 490 nm is also included in dried brown scales of onion bulbs (*37*). These results suggest that quercetin, which is formed by hydrolysis of quercetin glucosides in onion scales during aging (*22*), can be oxidized to compound **1** and that compound

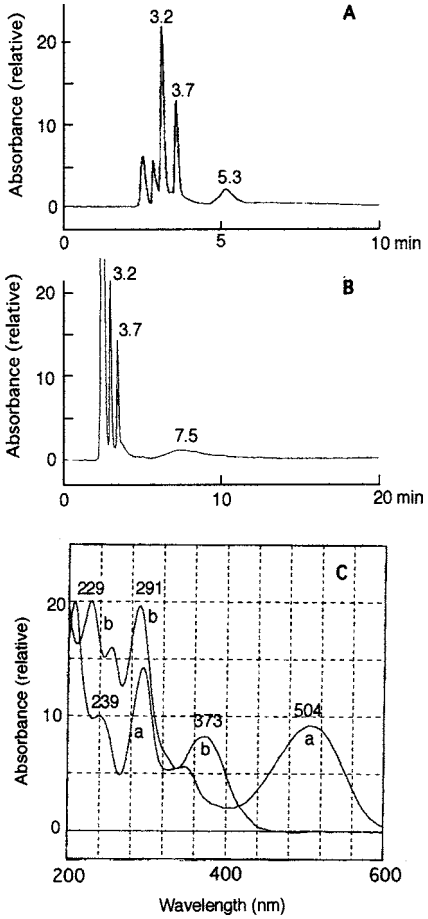

Figure 8. Separation of products formed by horseradish peroxidase-dependent oxidation of compound **1**: (**A**) HPLC profile after oxidation of compound **1**; (**B**) HPLC profile after reduction of the oxidation products by 0.2 mM ascorbic acid; (**C**) absorption spectra of components with retention times of 5.3 (spectrum a) and 7.5 min (spectrum b) in panels **A** and **B**, respectively.

1 can be transformed further to the above phenolic acids and red component during the aging. A red component was formed by the oxidation of compound **1** by horseradish peroxidase but not by peroxidase in saliva (**Figure 7**). This result suggests that the oxidation paths of compound **1** were different between the two peroxidases.

In this study, it is presented that transformation of nitrite to nitrogen dioxide radicals by peroxidase in saliva was preferential under acidic conditions. This result suggests that the formation of the radicals can proceed at sites where the pH is decreased by proliferation of acid-producing bacteria. In addition, the formation of nitrogen dioxide radicals may be increased after the ingestion of nitrate-rich foods because nitrate is reduced to nitrite in the oral cavity (*4*, *5*). Reactive nitrogen species such as nitrogen dioxide radicals can also be formed from nitric oxide in the oral cavity because certain oral bacteria can reduce nitrite to nitric oxide (*38*−*41*). Not only quercetin but also the oxidation product of quercetin, which was identified as compound **1**, seemed to be oxidized by nitrogen dioxide radicals. It has been reported that the concentration of quercetin in the precipitate of saliva, which mainly contains epithelial cells of the oral cavity, decreases from ∼5 to nearly 0 μM during 4 h after the ingestion of onion soup (*6*). From theses results, we can deduce that quercetin can effectively inhibit nitration in the oral tissues by scavenging reactive nitrogen species to minimize their risk to the health of the oral cavity.

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