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The presence of 4-hydroxyphenylacetic acid in human saliva and the possibility of its nitration by salivary nitrite in the stomach

Umeo Takahama[a,*], Takayuki Oniki[a], Hideaki Murata[b]

[a] *Kyushu Dental University, Kitakyushu 803-8580, Japan*
[b] *CSC, Shimadzu, Kyoto 604-8511, Japan*

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Abstract Human saliva contained 4-hydroxyphenylacetic acid (HPA) (2–10 μM) and nitrite (60–300 μM). HPA was nitrated to 4-hydroxy-3-nitrophenylacetic acid (NO_2HPA) when HPA and sodium nitrite were mixed at pH 1.0. NO_2HPA was also formed when saliva was incubated under acidic conditions. These results suggest that salivary HPA is nitrated to NO_2HPA when saliva is swallowed into the stomach.

Key words: 4-Hydroxyphenylacetic acid; 4-Hydroxy-3-nitrophenylacetic acid; Nitration; Nitrous acid; Saliva

1. Introduction

Phenolics are, in general, intermediates in the microbial degradation of aromatic amino acids [1–3]. In the oral cavity, microorganisms may also deaminate aromatic amino acids to the corresponding phenolics. In addition, there is a possibility that salivary glands secrete phenolic compounds, because mammals can produce phenolics during the metabolism of aromatic amino acids [4]. At present, as far as the authors know, there are no reports available on the presence of phenolics and on their function in saliva. Saliva contains nitrite ($pK_a = 3.3$), which can decompose to various nitrogen oxides including nitric oxide [5,6] and can nitrate phenolics [7] under acidic conditions. Therefore, if saliva contains phenolics, the phenolics may be nitrated when saliva is swallowed into the stomach. This communication deals with the identification and quantification of 4-hydroxyphenylacetic acid (HPA) in human saliva and with the nitration of HPA to 4-hydroxy-3-nitrophenylacetic acid (NO_2HPA) by salivary nitrite under acidic conditions.

2. Materials and methods

2.1. Reagents

NO_2HPA was obtained from Aldrich Japan (Tokyo, Japan). HPA and Griess-Romijn nitrite reagent were from Wako Pure Chem. Ind. (Osaka, Japan).

2.2. Saliva preparation

Whole mixed saliva (10 ml) was collected from the staff members of the Kyushu Dental University (five persons), who had no inflammation in their oral cavities, by chewing parafilm at about 9 a.m. The collected saliva was centrifuged at $20\,000 \times g$ for 5 min at 4°C to remove cellular components. Immediately after the centrifugation, the nitrite concentration of the supernatant was determined with Griess-Romijn nitrite reagent. The mixture to determine the concentration contained 0.1 ml of 1% Griess-Romijn nitrite reagent, 0.05 ml of saliva and 0.85 ml of 50 mM KCl–HCl buffer (pH 1.5). The concentration of nitrite was determined from the absorption at 540 nm after 15 min of incubation at 35°C.

2.3. Identification of salivary phenolics

Immediately after the addition of 2.5 M HCl (60 μl) to centrifuged saliva (3 ml) (final pH, about 1.6), the HPA in saliva was extracted with 5 ml of ethyl acetate twice. The ethyl acetate extracts were combined and ethyl acetate was evaporated with a rotary evaporator at about 30°C. The residue was dissolved in 0.1 ml of methanol for analysis by high performance liquid chromatography (HPLC). When HPA was detected using a spectrophotometric detector with a photodiode array (SPD-M10A, Shimadzu, Kyoto, Japan), a Shim-pack CLC-ODS column (6 mm i.d. × 15 cm) (Shimadzu) was used. The mobile phase (flow rate, 1 ml min^{-1}) was a mixture of methanol and 25 mM KH_2PO_4 (1:6, v/v). HPA was quantified from the peak areas on chromatograms at 280 nm. No detectable nitration of HPA was observed during the extraction of HPA from saliva.

HPA in saliva was also studied using a Shim-pack VP-ODS column (2 mm i.d. × 15 cm, Shimadzu) combined with a mass spectrometric detector (LCMS-2010, Shimadzu). The mobile phase (flow rate, 0.2 ml min^{-1}) was a mixture of 5 mM dibutylammonium acetate and acetonitrile; the concentration (v/v) of acetonitrile was linearly increased from 10% (0 min) to 70% (15 min).

2.4. Nitration of HPA

HPA was nitrated at 25°C in a reaction mixture (1 ml) that contained $NaNO_2$ and HPA in 50 mM KCl–HCl buffer (pH 1.0). After incubation for defined periods, the reaction mixture was extracted with 2 ml of ethyl acetate twice and the ethyl acetate extracts were combined. Ethyl acetate was evaporated with a rotary evaporator, and the residue was dissolved in 0.3 ml of mobile phase used for HPLC. A Shim-pack CLC-ODS column (6 mm i.d. × 15 cm) was used to separate NO_2HPA and the compound was detected using a spectrophotometric detector with a photodiode array (SPD-M10A). A mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v; pH 4.5) served as the mobile phase and the flow rate was 1 ml min^{-1}.

Centrifuged saliva (3 ml) was incubated at 35°C at pH 1.6. The pH was adjusted by adding 2.5 M HCl. The NO_2HPA that formed in the saliva was extracted with 5 ml of ethyl acetate twice. NO_2HPA in the ethyl acetate extract was not separated from other salivary components when the above mobile phase (pH 4.5) was used. Then, we used a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v; pH 3.0) as the mobile phase (flow rate, 1 ml mim^{-1}) to separate NO_2HPA from other salivary components. The pH was adjusted by adding 1 M H_3PO_4. NO_2HPA was quantified from the peak areas on chromatograms at 360 nm.

*Corresponding author. Fax: (81)-93-581 3202.
E-mail address: takahama@kyu-dent.ac.jp (U. Takahama).

Abbreviations: HPA, 4-hydroxyphenylacetic acid; NO_2HPA, 4-hydroxy-3-nitrophenylacetic acid

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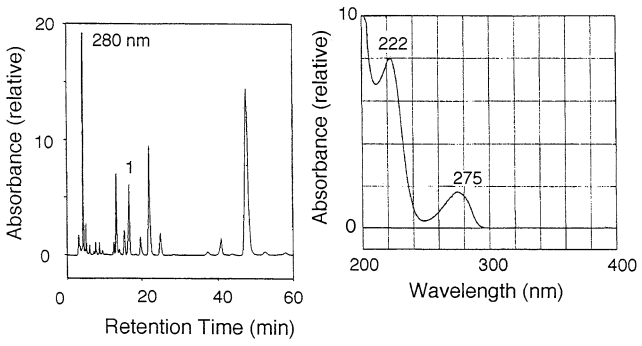

Fig. 1. A typical HPLC profile of ethyl acetate extract of centrifuged saliva. Left: HPLC profile. Peak 1, HPA. Right: Absorption spectrum of peak 1. The mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (1:6, v/v).

3. Results and discussion

Fig. 1 (left) shows a typical HPLC profile of ethyl acetate extract of centrifuged saliva. The retention time of peak 1 (17.8 min) was identical to that of authentic HPA. The absorption spectrum of peak 1 (Fig. 1, right), which had peaks at 222 and 275 nm, was also identical to that of authentic HPA. A component similar to HPA was separated in an HPLC system used for a liquid chromatography/mass spectrometer system and the retention time was 6.0 min. Mass numbers (*m*/*z*) of the component were 151 (M−H), 211 (M−H+acetic acid) and 303 (2M−H) when ionized by the electrospray method and the values were identical to those of authentic HPA. These results indicate that HPA is a component of saliva. Table 1 shows the concentrations of HPA of 10 saliva preparations from five persons (two saliva preparations from each person collected on different days) (measurement 1) and six saliva preparations collected from one individual on different days (measurement 2). Although there were deviations, the values were between 2 and 10 μM. It is unclear, at present, whether salivary HPA is derived from oral microorganisms, salivary glands or others.

There are reports that HPA is nitrated by oxidation intermediates of nitrite [8–10]. When HPA (30 μM) was incubated with sodium nitrite (0.2 mM) in 50 mM KCl–HCl buffer (pH 1.0), a component (peak 2) was detected by HPLC (Fig. 2A). The retention time (9.2 min) and absorption spectrum (peaks; 216, 280 and 360 nm) were identical to those of authentic NO_2HPA. When the concentration of NO_2HPA was plotted as a function of incubation time in the presence of 0.2 mM $NaNO_2$ and 10, 30 or 100 μM HPA, NO_2HPA was formed biphasically, namely, an initial rapid formation (within 1 min) was followed by a second steadier formation (not shown). The latter was nearly linear over 60 min of incubation. The NO_2HPA that formed after 1 and 30 min of incubation was proportional to the concentrations of $NaNO_2$ (Fig. 2B) and

Table 1
Concentrations of HPA and nitrite in saliva

	HPA (μM)	Nitrite (mM)
Measurement 1[a]	4.8 ± 2.1 (n = 10)	0.16 ± 0.10 (n = 10)
Measurement 2[b]	6.2 ± 1.7 (n = 6)	0.18 ± 0.11 (n = 6)

Values are means ± S.D.
[a]Saliva from five persons (two saliva preparations for each).
[b]Saliva from one person on different days.

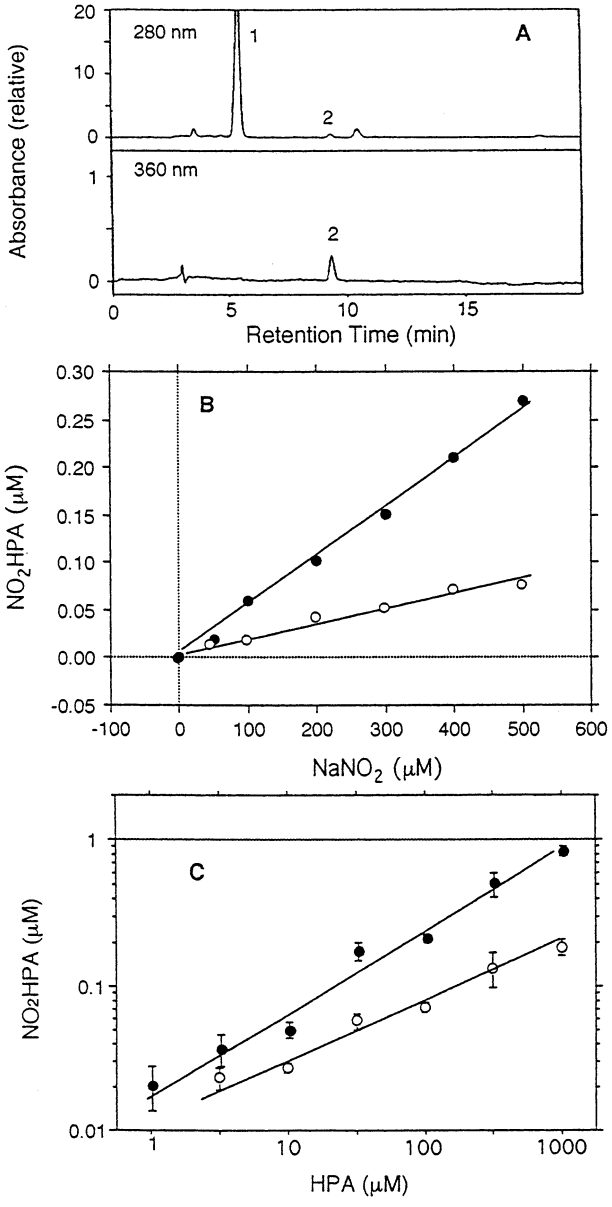

Fig. 2. Formation of NO_2HPA from HPA and sodium nitrite under acidic conditions. A: A typical HPLC profile of NO_2HPA. The reaction mixture (1 ml) contained 0.2 mM $NaNO_2$ and 30 μM HPA in 50 mM KCl–HCl (pH 1.0). HPA and NO_2HPA were extracted with ethyl acetate after 30 min of incubation. Peak 1, HPA; peak 2, NO_2HPA. B: NO_2HPA formation as a function of $NaNO_2$ concentration. The reaction mixture (1.0 ml) contained 100 μM HPA and various concentrations of $NaNO_2$ in 50 mM KCl–HCl buffer (pH 1.0). C: NO_2HPA formation as a function of HPA concentration. The reaction mixture (1.0 ml) contained 0.2 mM $NaNO_2$ and various concentrations of HPA in 50 mM KCl–HCl buffer (pH 1.0). The reaction mixtures for B and C were extracted with ethyl acetate after 1 and 30 min of incubation to quantify HPA and NO_2HPA by HPLC. Open circles, 1 min; closed circles, 30 min. The data plots represent means of two (B) and three (C) separate experiments. Bars in C are S.D. (n = 3).

HPA (Fig. 2C). The concentration of NO_2HPA that formed after 30 min of incubation increased as the pH was reduced from 5 to 1 (data not shown).

Since saliva contains about 5.5 μM HPA (Table 1) and about 0.17 mM nitrite (Table 1 and Ferguson [11]) on average, there is a possibility of transformation of salivary HPA

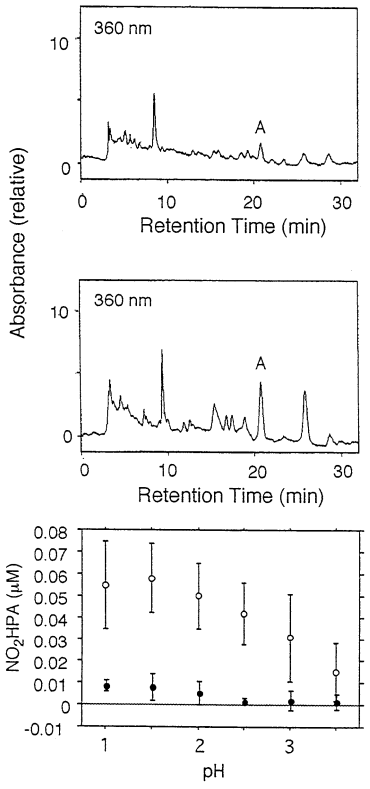

Fig. 3. Formation of NO_2HPA in saliva. Top and Middle: HPLC profiles. Centrifuged saliva was incubated for 30 min at pH 1.6 in the presence and absence of $NaNO_2$. Top, without $NaNO_2$; middle, with 1 mM $NaNO_2$. Peak A, NO_2HPA. Bottom: Effects of pH. Centrifuged saliva was incubated for 30 min at various pHs. Closed circles, without $NaNO_2$; open circles, with 1 mM $NaNO_2$. The data plots represent means of 3–4 experiments (bars, S.D.).

to NO_2HPA when saliva is swallowed into the stomach. Then, we studied whether NO_2HPA is formed or not in saliva under acidic conditions. When centrifuged saliva was extracted with ethyl acetate immediately after the acidification, no NO_2HPA was detected. In the acidified saliva that was incubated for 30 min at pH 1.6, a component (peak A in Fig. 3, top) was detected and the retention time (about 21 min) and absorption spectrum (peaks; 215, 275 and 357 nm) were identical to those of authentic NO_2HPA. The peak area increased on the addition of 1 mM $NaNO_2$ (Fig. 3, middle). Table 2 summarizes the amounts of NO_2HPA that formed after incubation of centrifuged saliva for 30 min at pH 1.6 with and without 1 mM $NaNO_2$. The values were 0.004–0.018 μM in the absence of $NaNO_2$ and 0.04–0.11 μM in the presence of 1 mM $NaNO_2$. Fig. 3 (bottom) shows the effects of pH on the formation of NO_2HPA in the presence and absence of externally added nitrite. The formation of NO_2HPA was increased as the pH was reduced from 3.5 to 1. It has been reported that nitrite-dependent nitric oxide formation increases as the pH is decreased from 5 to 1 [5,6]. The results in Fig. 3 and Table 2 imply that when saliva was swallowed into the stomach, NO_2HPA formed and that the formation increased as the concentration of nitrous acid increased.

Table 2
Formation of NO_2HPA during incubation of saliva at pH 1.6

	NO_2HPA in saliva (μM)		
Incubation time (min)	0	30	30 (+1 mM $NaNO_2$)
Measurement 1[a]	–[c]	0.012 ± 0.006	0.073 ± 0.034
Measurement 2[b]	–[c]	0.011 ± 0.009	0.077 ± 0.025

Values are means ± S.D.
[a]Saliva from five different persons ($n = 5$).
[b]Saliva from one person on different days ($n = 6$).
[c]Below detection.

Nitration of free [12,13] and proteinic [13,14] tyrosine by nitrous acid has been reported. Reactive nitrogen species like NO, NO_2 [7,15] and N_2O_3 [16] participate in the nitration of phenolics including tyrosine. Since NO is formed from salivary nitrite in the stomach and NO_2 and N_2O_3 have been proposed as intermediates of the degradation of nitrous acid [5,6,13], it is possible that these nitrogen oxides participate in the nitration of salivary HPA in the stomach. One can therefore conclude that the significance of the nitration of HPA by salivary nitrite under acidic conditions is the scavenging of reactive nitrogen species that are formed from salivary nitrite in the stomach. Since horseradish peroxidase, myeloperoxidase and lactoperoxidase [10] and plasma polymorphonuclear leukocytes [9] can nitrite HPA, the nitration of salivary HPA by salivary peroxidase and salivary polymorphonuclear leukocytes is also possible. The quantification of salivary NO_2HPA may be a useful indicator of inflammation of the oral cavity. Studies along this line are under progress.

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