Original

The Effect of Green Tea Infusion on Acid Resistance of Hydroxyapatite

ヒドロキシアパタイトの耐酸性に及ぼす 緑茶抽出液の効果

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概要: 酢酸緩衝液 (pH 4.0) に対するヒドロキシアパタイトの耐酸性に及ぼす緑茶抽出液の効果を解析する目的で、緑茶抽出液中に存在する種々の無機および有機化合物の効果を検討した。ヒドロキシアパタイトへのフッ化物イオンの吸着量は液中のフッ化物イオン濃度に比例し、緑茶抽出液と NaF 水溶液に差異は認められなかった。すなわち、緑茶抽出液中のフッ化物イオンは、同じ pH 5.6において NaF 水溶液中のフッ化物イオンと同じ挙動を示すことが明らかとなった。一方、ヒドロキシアパタイトの耐酸性もまた処理溶液中のフッ化物イオン濃度に比例して強化されたが、フッ化物イオンの吸着量が同じであっても、緑茶抽出液の方が NaF 水溶液よりも効果が大きかった。その原因の一部は、緑茶抽出液中に含まれるアルミニウムイオンによることが明らかとなったが、緑茶抽出液中に存在するその他の物質は、ヒドロキシアパタイトの耐酸性強化に寄与しなかった。以上の結果より、ヒドロキシアパタイトの耐酸性向上に及ぼす緑茶抽出液の効果は、主としてフッ化物イオンとアルミニウムイオンによることが明らかとなったものの、それらのみでは緑茶抽出液の効果は理解できず、緑茶抽出液中に存在する極微量成分あるいはそれらと他の成分との相乗作用の寄与があることが示唆された。

Key words: Hydroxyapatite, Acid resistance, Fluoride, Aluminum, Green tea infusion

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Introduction

Tea has long been known as an important source of dietary fluoride¹⁾ and its anticariogenic effect has been demonstrated in a number of human²⁻⁴⁾ and animal studies⁵⁻⁸⁾.

Fluoride in tea can be taken up on the enamel surface and synthetic hydroxyapatite. Other constituents of tea also have influence on the fluoride uptake. Speirs reported the addition of Al (III) and Mn (II) to NaF solutions at pH 7.4 at concentrations corresponding to those determined in a tea infusion reduced the fluoride uptake by hydroxyapatite⁹⁾. However, the addition of tannic acid to Al (III) and Mn (II) neutralized their effect. His observations may be related to the chelating properties of polyphenols present in tea, in the same manner as Mn (II) to alkaloids^{10,11)}.

Friedman *et al.* studied the uptake of fluoride in tea by hydroxyapatite and the effect on its dissolution rate¹²⁾. They found that the fluoride uptake was related to fluoride concentration in the tea extracts. Although the fluoride content of hydroxyapatite samples treated with black tea was significantly higher than green tea-treated samples, the dissolution rates in acidic buffers were similar. The results suggest that the fluoride in tea may not be the single factor which affects the dissolution rate of hydroxyapatite.

On the other hand, Manly and Bibby investigated the effect of 147 substances on the acid resistance of powdered tooth enamel in respect to the topical application on human teeth¹³⁾. They found that 27 compounds such as $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, $\text{Pb}(\text{NO}_3)_2$, PbF_2 , ZnF_2 , BaF_2 , SrF_2 , $\text{Sn}(\text{NO}_3)_2$, *etc.* were more effective than 0.1 % NaF in reducing the acid dissolution rate, whereas FeF_3 , $\text{Al}(\text{NO}_3)_3$, AlF_3 , $\text{Ca}(\text{NO}_3)_2$, CaF_2 , $\text{Zn}(\text{NO}_3)_2$, $\text{Cu}(\text{NO}_3)_2$, $\text{Ba}(\text{NO}_3)_2$, $\text{Sr}(\text{NO}_3)_2$, AgF , *etc.* were less effective than 0.1 % NaF. The concentrations investigated (1 or 0.1 %) were very high compared to those in a green tea infusion. In general, enhanced fluoride uptake can be obtained with any polyvalent metal capable of forming strong complexes while simultaneously binding firmly to the apatite crystals^{14,15)}. A general characteristic of increasing acid resistance is believed to be the ability to form a relatively insoluble adherent and coherent layer of insoluble reaction product on the surface of the enamel¹⁶⁾. Therefore, cation-fluoride and cation-organic compound interactions must be taken into account. Dedhiya *et al.* reported that dramatically large synergistic effects with fluoride (1 ppm F) and strontium ($1 \times 10^{-8}\text{M}$) or magnesium ($1 \times 10^{-8}\text{M}$) completely inhibited the dissolution of hydroxyapatite^{17,18)}. However, Sr (II) and Mg (II) are not present in a green tea infusion at such high concentrations.

The aim of the present study was to investigate how fluoride in a green tea infusion (pH 5.6) behaves in comparison with fluoride in a NaF solution at pH 5.6 and what ions or compounds other than fluoride present in a green tea infusion at the similar concentrations to those in a green tea infusion affect the acid-susceptibility to the dissolution of synthetic hydroxyapatite. High concentrations of potassium, magnesium, phosphate, calcium, and aluminum and significant amounts of manganese, fluoride, iron, and zinc are present in a green tea infusion and many other metals such as copper, nickel, cobalt, lead, selenium, antimony, and cadmium are present in trace amounts¹⁹⁾. As organic materials, tannic acid, catechin, caffeic acid, quercetin, or theanine are present in large amounts²⁰⁾. In this study, inorganic cations and organic compounds investigated were KNO_3 , CaCl_2 , MgCl_2 , $\text{Zn}(\text{CH}_3\text{COO})_2$, MnCl_2 , FeCl_2 , FeCl_3 , AlCl_3 , NaF, tannic acid, *d*-catechin, caffeic acid, quercetin, and theanine.

Materials and methods

Materials

Hydroxyapatite (HA , $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$: for HPLC, 75-150 μm Lot. No. SAF 7207) and NaF standard solution (100 ppm as F) were purchased from Wako Pure Chemicals (Japan) and Orion Research (USA), respectively. The other reagent-grade chemicals were obtained from Nacalai Tesque (Japan).

Green tea (coarse) used in this study was produced in Uji (Japan). Green tea was infused in 100 ml of distilled water at a temperature of 90 to 93 °C for an appropriate time, then filtered. The pH of the tea infusion was 5.6. Concentrations of the infusion were controlled by varying the amounts of green tea (2–5 g) and time of infusion (10–20 min).

Reconstructed “inorganic tea infusion” contained 50 µg F, 100 µg Mg, 60 µg Ca, 100 µg Al, 0.6 µg Zn, 50 µg Mn, and 1.0 µg Fe/2 ml. Reconstructed “organic tea infusion” contained tannic acid 10 mg, *d*-catechin 10 mg, caffeic acid 1 mg, quercetin 1 mg, and theanine 5 mg/2 ml. Reconstructed “inorganic plus organic tea infusion” contained 50 µg F, 100 µg Mg, 60 µg Ca, 100 µg Al, 0.6 µg Zn, 50 µg Mn, 1.0 µg Fe, tannic acid 10 mg, *d*-catechin 10 mg, caffeic acid 1 mg, quercetin 1 mg, and theanine 5 mg/2 ml. These concentrations were similar to or slightly higher than the concentrations in the green tea infusion measured by ourselves. These three reconstructed solutions were adjusted to pH 5.6 in 0.125 M potassium phosphate buffer.

Instruments

Fluoride analysis was performed with an Orion combination fluoride electrode (model 96-09) attached to an Orion Ionanalyzer (model 901; Orion Research Inc., Cambridge, MA). A UV-Visible recording spectrophotometer (UV-260; Shimadzu, Japan) was used for determination of phosphate by the molybdenum blue method. Total concentration of K, Al, Ca, Fe, and Zn was determined with an atomic absorption spectrophotometer (AA-660; Shimadzu, Japan) and Mn and Mg were analyzed by ICPS (ICPS-1000 II; Shimadzu, Japan) after the tea infusion was treated in a mixture of nitric acid and perchloric acid. Electron microscopic analysis of hydroxyapatite was performed with a Hitachi S-570 electron microscope (20 kV, magnificent ×500). Samples were coated with gold before analysis.

Treatment of HA with various materials

The experimental design is illustrated in Fig. 1. Typical treatment procedures of powdered hydroxyapatite with a NaF treatment-solution were as follows: hydroxyapatite (100 mg) was added to a 2 ml solution of 0.1 M sodium phosphate buffer (pH 5.6) containing NaF^{21,22}. After a 10 min shaking in a 37 °C constant temperature shaking bath, the mixture was centrifuged (3000 rpm, 3 min). The residual apatite was rinsed twice with 10 ml of distilled water, then dried at 37 °C for 24 h.

Materials other than NaF and a green tea infusion used for treatment of HA were K (I), Mg (II), Al (III), Ca (II), Mn (II), Zn (II), Fe (II), Fe (III), tannic acid, *d*-catechin, caffeic acid, quercetin, or theanine in 0.1 M sodium phosphate buffer (pH 5.6) and their mixtures as the reconstructed tea infusions. Treatment of hydroxyapatite with an Al (III) solution was performed at pH 2.3 by using a standard aluminum solution for atomic absorption analysis (1000 ppm as Al) without phosphate buffer in order to avoid the precipitation of Al(OH)₃ at pH 5.6.

Figure. 1 Experimental design.

Figure. 2 Fluoride uptake by hydroxyapatite from NaF solutions and tea infusions. After HA(100 mg) was treated with 2 ml of NaF (○) or tea (●) solutions for 10 min at 37 °C, it was completely dissolved in 5 ml of 1.2M HCl, and the fluoride content was determined with a fluoride ion electrode.

HV, 0.45 µm), then washed twice with 10 ml of distilled water and dried at 37 °C for 24 h. The amount of dissolved hydroxyapatite was calculated from the phosphate concentration in the filtrate, which was determined colorimetrically by the molybdenum blue method with hexaammonium heptamolybdate²³). Acid resistance of hydroxyapatite treated with H₂O instead of fluoride solutions served as a control.

Acid resistance of hydroxyapatite treated with 0.1 M sodium phosphate buffer (pH 5.6) was 1.0.

Results

Fluoride uptake by HA

Figure 2 shows the dependency of fluoride uptake on the fluoride concentration of the NaF treatment solution in 0.1 M sodium phosphate buffer (pH 5.6). A good linear correlation between fluoride uptake by hydroxyapatite (0–34 µg F) and the concentration of the NaF treatment solution was observed in the range of 0–200 µg F/2 ml. Fluoride uptake from the green tea infusion (21.8 and 56.6 µg F/2 ml) lies on the same line with that of NaF. The regression line is represented as a linear equation, $y = 0.17x + 0.41$ ($r = 0.999$), where y is the amount of fluoride uptake and x is the fluoride concentration of NaF treatment solutions; thus 17 % of fluoride in the solution was adsorbed onto hydroxyapatite.

Acid resistance

The amount of phosphate dissolved in acetate buffer (pH 4.0) from hydroxyapatite treated with various concentration of a NaF solution or green tea infusion are summarized in Table 1. A linear relationship between calculated acid resistance and fluoride content in treatment solutions used was observed below the concentration of 200 µg F/2 ml as shown in Fig. 3. In the case of the NaF solution, the correlation is represented as $y = 0.0060x + 1.02$ ($r = 0.993$), where y is the acid resistance and x is the fluoride concentration of NaF treatment solutions. The tea infusion also showed a linear relationship which was represented as $y = 0.0174x + 1.07$ ($r = 0.990$). These correspond to 30 and 51 % reduction from the control by the treatment with the NaF solution (56.6 µg F/2 ml) and the green tea infusion containing fluoride (56.6 µg F/2 ml),

Determination of fluoride uptake by HA

Hydroxyapatite treated with a NaF treatment solution or a green tea infusion was completely dissolved in 5 ml of 1.2M HCl at 25 °C. After dilution of 100 µl of the resultant solution with 10 ml of 0.5 M sodium citrate buffer (pH 6.0), fluoride concentration was determined with the fluoride electrode^{9,23,24}.

Determination of Acid resistance

The following equation was used for the calculation of the acid resistance:

Acid resistance = P (from control sample) / P (from sample), where P is the amount of phosphate dissolved into acetate buffer (pH 4.0). Sixty percent reduction of the dissolution rate of hydroxyapatite compared with a control corresponds to 2.5 of the acid resistance.

Hydroxyapatite treated with various materials was shaken in 10 ml of 0.4M sodium acetate buffer (pH 4.0) at 25 °C. After 5 min, the HA was immediately filtered by Millipore filter (Millex-

Table 1 Phosphate dissolution from hydroxyapatite treated with NaF solution and tea infusion in acetate buffer and acid resistance^{a)}

	Fluoride Contents ($\mu\text{g F}/2\text{ml}$)	Dissolved Phosphate ($\mu\text{g P}/100\text{mg HA}^{\text{b)}$)	Acid resistance ^{c)}
Control	0.0	8.05	1.00
NaF	4.0	8.05	1.00
	8.0	8.05	1.00
	10.0	7.42	1.08
	20.0	6.82	1.18
	40.0	6.36	1.27
	50.0	6.29	1.28
	56.6	5.67	1.42
	100.0	5.39	1.66
	200.0	3.69	2.18
	400.0	3.01	2.67
	600.0	2.65	3.04
Tea	800.0	2.61	3.08
	1000.0	2.52	3.19
	4.8	6.65	1.21
	12.4	6.39	1.26
	21.8	5.29	1.52
	40.8	4.52	1.78
	56.6	3.97	2.03

^{a)} HA (100mg) treated with NaF or tea, were dissolved in 10 ml of 0.4M acetate buffer (pH 4.0) for 5 min at 25°C.

^{b)} HA : hydroxyapatite.

^{c)} Acid resistance = $P(\text{control HA}) / P(\text{treated HA})$: see in text.

respectively. The effect of the green tea infusion was consistently higher than that of the NaF solution.

Effects of other inorganic materials, KNO_3 , CaCl_2 , MgCl_2 , $\text{Zn}(\text{CH}_3\text{COO})_2$, MnCl_2 , FeCl_2 , FeCl_3 , AlCl_3 , and NaF are summarized in Table 2 together with the concentrations found in a green tea infusion. Cations. K (I), Ca (II), Mg (II), Zn (II), Mn (II) did not affect the acid resistance at a concentration comparable with that in the tea infusion. Although Fe (II) showed a great increase in the acid resistance at a high concentration (2.83 at 1.2mg Fe (II)/2ml), it was only 1.09 at the level of a green tea infusion (1.09 at 4.0 μg Fe (II)/2ml). Among the inorganic materials studied, only Al (II) showed a large positive effect. The effect of Al (III) on the acid resistance was 1.23 at 100 μg Al/2ml. As the aluminum concentration increased, the acid resis-

Figure. 3 Acid resistance of hydroxyapatite treated with NaF (O) and tea (●) solutions. After HA (100mg) was treated with NaF (O) or tea (●) solutions for 10min at 37°C, it was shaken in 10 ml of 0.4 M acetate buffer (pH 4.0) for 5min at 25°C, and the P content in the supernatants was determined photometrically.

Table 2 Acid Resistance of Hydroxyapatite treated with Various Inorganic Materials^{a)}

Materials ^{b)}	Conc. in green tea	Acid resistance ^{c)}
KNO ₃ (3mg K)	1.36mg K	1.00
CaCl ₂ (60μg Ca)	59.0μg Ca	1.00
MgCl ₂ (100μg Mg)	96.2μg Mg	1.03
Zn(CH ₃ COO) ₂ (150μg Zn)	0.6μg Zn	1.03
MnCl ₂ (50μg Mn)	41.8μg Mn	1.05
FeCl ₂ (4.0μg Fe)	1.0μg Fe	1.09
FeCl ₂ (1.2mg Fe)		2.83
FeCl ₃ (1.2mg Fe)		1.11
AlCl ₃ (100μg Al)	101.2μg Al	1.23
NaF (56.6μg F)	56.6μg F	1.42

^{a)} HA (100mg) treated with NaF or various substances in 0.1 M sodium phosphate (2 ml, pH 5.6) were treated in 0.4M acetate buffer (10 ml, pH 4.0)

^{b)} Amounts in 2 ml of the treatment solution.

^{c)} Acid resistance = P (control HA)/P (treated HA).

Figure. 4 Acid resistance of hydroxyapatite treated with AlCl₃ (O) and tea (●) solutions. After HA (100mg) was treated with AlCl₃ or tea infusions for 10min at 37°C, it was dissolved in 10ml of 0.4M acetate buffer (pH 4.0) for 5min at 25°C, and the P content in the supernatant was determined photometrically.

tance also increased linearly as shown in Fig. 4.

Organic substances such as tannic acid, *d*-catechin, caffeic acid, quercetin, or theanine, had no or very little effect on the acid resistance as summarized in Table 3. Even when NaF was added, a synergistic effect was small.

Reconstructed "inorganic tea infusion", "organic tea infusion", and "inorganic plus organic tea infusion" produced acid resistance of 1.55, 1.06, and 1.67 respectively, as summarized in Table 4.

Electron microscopic analysis

SEM pictures (Fig. 5) show that dissolution was more inhibited on hydroxyapatite treated with a green tea infusion than that treated with an NaF solution.

Discussion

Figure 2 shows that fluoride uptake was linearly related to fluoride concentration of an NaF solution and a green tea infusion, which

indicates that the fluoride in a green tea infusion behaves in the same manner as the ionic fluoride in an NaF solution in 0.1 M phosphate buffer (pH 5.6). This is somewhat in contrast to the conclusions of the study done by Speirs⁹⁾. Speirs reported that the fluoride uptake by hydroxyapatite was consistently less from tea infusions than from an NaF solution of the matched fluoride concentration with tea at pH 7.4, whereas at the natural pH of a tea infusion (about 6.0 or less), fluoride uptake from the tea infusion increased to an extent similar to that from the NaF solution at pH 7.4. He concluded that

Table 3 Acid Resistance of Hydroxyapatite treated with Various Organic Materials with or without NaF^{a)}

Materials	Acid resistance ^{b)}	
	without NaF	with NaF (20 μ g F)
None		1.19
Tannic acid (10mg)	1.03	1.27
Catechin (10mg)	1.00	1.16
Caffeic acid (1mg)	1.00	1.18
Theanine (5mg)	1.03	1.23
Quercetin (1mg)	1.02	1.25

^{a)} HA (100mg) treated with NaF and/or various materials was dissolved in 0.4 M acetate buffer (10 mL, pH 4.0) for 10 min at 37°C.

^{b)} Acid resistance = P (control HA)/P (treated HA).

Table 4 Acid Resistance of Hydroxyapatite treated with Reconstructed Tea Infusions

Reconstructed Tea infusion	Acid resistance ^{a)}
Inorganic (K, Mg, Ca, Zn, Mn, Fe, Al, NaF)	1.55
Organic (Tannic acid, Catechin, Caffeic acid, Quercetin, Theanine)	1.06
Inorganic+Organic Tea Infusion	1.67 2.03

^{a)} Acid resistance = P (control HA)/P (treated HA).

Figure. 5 Scanning electron microphotograph showing the appearance of hydroxyapatite (magnification x 500). A: untreated HA; B: HA treated with H₂O; C: HA treated with NaF; D: HA treated with tea. After HA (100 mg) was treated with NaF or tea solutions for 10 min at 37°C, it was shaken in 10 mL of 0.4 M acetate buffer (pH 4.0) for 5 min at 25°C. The residual HA was dried and observed with an SEM.

the behavior of the various fluoride fractions (ionized, complexed, and bound fluorides) in tea may be different between pH 5.35–5.4 and pH 7.4. Our results indicate that at pH 5.6 the amount of adsorbed fluoride on hydroxyapatite is not affected by other components in a green tea infusion, that is, at pH 5.6 the fluoride fraction in the tea infusion is present in the same ionized form as in the NaF solution.

As shown in Fig. 3, the effect of NaF on the acid resistance was linearly related to the fluoride concentration in the NaF solution at 0–200 $\mu\text{g F}/2\text{ ml}$. The effect of the green tea infusion on the acid resistance was also linearly related to the fluoride concentration of the green tea infusion at 0–56.6 $\mu\text{g F}/2\text{ ml}$. However, the green tea infusion showed a much larger effect than the NaF solution regardless of the fact that fluoride uptake was the same.

SEM clearly showed that the surface of hydroxyapatite treated with a green tea infusion remained unchanged even after it was treated in the acetate buffer (pH 4.0), whereas hydroxyapatite treated with an NaF solution had more rough surface. This result also indicates that some materials other than fluoride in green tea infusion are responsible for the increase in the acid resistance of hydroxyapatite.

Friedman *et al.* reported that although the fluoride concentration of black tea was about five times higher than that of green tea and that fluoride uptake by hydroxyapatite from black tea was also five times higher than that from green tea, the dissolution rates in acidic buffers were similar¹². They suggested that the fluoride content might not be the only factor which governs the dissolution of hydroxyapatite treated with black or green tea infusions. Our present results showed that as the fluoride uptake increased, the acid resistance also increased linearly. However, this does not mean that fluoride is the only factor which governs the acid resistance of hydroxyapatite, because the ratio of fluoride to other components might be always the same. Although Friedman *et al.* did not compare the effects of tea infusions with those of NaF solutions of the matched fluoride concentrations¹², it is possible that the effect of fluoride had been already leveled off at about 200 $\mu\text{g F}/2\text{ ml}$ fluoride uptake.

In order to discover the difference between a green tea infusion and an NaF solution, it is important to study the effects of other components present in a green tea infusion at the matched concentrations.

As clearly shown in Fig. 3, Al (III) was an effective component in a green tea infusion for producing acid resistance. To our knowledge, a positive effect of Al (III) at such a low concentration has not been reported. The other metallic cations such as K (I), Ca (II), Mg (II), Zn (II), Mn (II), Fe (II) and (III), however, did not show a significant effect on the acid resistance at the tea concentrations, although Fe (II) and (III) in high concentrations showed large effects as shown in Table 2. Table 3 shows that organic compounds such as tannic acid, *d*-catechin, caffeic acid, quercetin, and theanine, did not have any effect on the acid resistance at concentrations matched with the green tea infusion. Even when NaF was added to the treatment solution, a synergistic effect was not observed.

Our results showed that components of a green tea infusion other than fluoride and Al (III) studied here were ineffective for the inhibition of hydroxyapatite dissolution. However, the effect of a green tea infusion on the acid resistance can not be fully explained only by fluoride and Al (III), because the green tea infusion with 56.6 $\mu\text{g F}/2\text{ ml}$ gives acid resistance of 2.03 and the NaF solution with 56.6 $\mu\text{g F}/2\text{ ml}$ and the Al (III) solution with 100 $\mu\text{g Al}/2\text{ ml}$ give 1.42 and 1.23, respectively. The value for tea is significantly higher than the sum of the values for fluoride and Al (III).

As mentioned in the introduction, the combination of low levels of cation-fluoride and cation-organic compound may significantly reduce dissolution of hydroxyapatite. In order to find the unknown reason why increases acid resistance, we examined effects of several reconstructed tea infusions on the dissolution of hydroxyapatite. The results summarized in Table 4 show that none of the reconstructed "tea infusions" produced acid resistance in hydroxyapatite similar the green tea infusion. The observed effects seemed

only the sum of the effects of fluoride and Al (III). Although at present we do not fully understand the chemistry of a green tea infusion, the present results indicate that very minor components in a green tea infusion and/or their interactions with other components must be important for increasing acid resistance of hydroxyapatite.

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