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Precipitation of octacalcium phosphates on artificial enamel in artificial saliva

Y. Sato · T. Sato · M. Niwa · H. Aoki

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Abstract The natural saliva samples were collected from 30 Japanese men and women aged between 20 and 30, and the inorganic components in the saliva were analyzed chemically. Artificial saliva (AS) was prepared based on the chemical analysis using chemical reagents. The calcium/phosphate molar ratio of the AS was 0.28 with pH = 7.0. Artificial enamel (AE) was prepared by sintering hydroxyapatite powder at 1,200°C. The AE was placed in the AS at 37°C. XRD, SEM and EDX investigated precipitation on the AE. The precipitation was always identified as being OCP. As decrease in the pH of the surface of the AE and increase in the concentration of phosphate ions were observed by chemical analysis, it was concluded that the OCP occurs by the pH decrease and phosphate ion concentration increase on the surface of AE.

1 Introduction

The interaction of saliva with enamel has been investigated from the viewpoints of digestive and chemical buffering actions and of calcification. Among these, calcification, i.e., the precipitation of calcium phosphate, has been the major interest and it has been the topic of many research papers. In 1983, Gelhard et al. reported that remineralization was observed when human front-tooth enamel that had been slightly decalcified in a 0.1 M potassium acetate solution at pH = 5.0 was put into artificial saliva (AS) containing mucin and carboxymethyl-cellulose. They concluded that the carboxymethyl-cellulose and mucin had promoted the remineralization [1]. In 1998, Amaechi et al. reported that bovine tooth enamel dissolved in orange juice at pH = 3.85 ± 0.05 could undergo remineralization in either natural saliva or AS [2–4]. In 2001, Eisenburger et al. reported that human molar enamel dissolved in 0.3% citric acid at pH = 3.2 underwent mineralization after 6 h in an AS [5]. Most of these remineralization studies were carried out using natural tooth enamel and natural saliva, and therefore, varying results were observed, due to the individual differences in the age or sex of the donor of the teeth and saliva samples. The composition of saliva changes, depending on the type of saliva glands used, and the time of extraction. And few in these report described about OCP precipitation.

To circumvent these variables, we carried out precipitation experiments using an AS without mucin and carboxymethyl-cellulose, and artificial enamel (AE). The AE was made of the densely sintered hydroxyapatite (HA) that had almost the same composition as natural enamel. The AE had no preferred orientation of the HA crystals, whereas natural enamel has a preferred parallel orientation along the *c*-axis of the hexagonal apatite structure. The AS was prepared by mixing inorganic chemical reagents, and was based on analytical values from saliva collected from 30 Japanese men and women aged between 20 and 30. The composition of the AS was within the range of previously reported values [6–8]. The AE was soaked in the AS, and precipitation on the AE was identified using X-ray diffraction (XRD), scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy (EDX). Furthermore, modified metastable AS samples with calcium/phosphate (Ca/P) molar ratios of 0.64 and 1.93, were prepared by adding calcium ion to the AS. The modified AS solutions were subsequently used in precipitation experiments. The precipitation on the AE in

Y. Sato (✉) · T. Sato · M. Niwa · H. Aoki
The Nippon Dental University, School of Dentistry at Tokyo,
Department of Preventive and Community Dentistry
e-mail: yuji21@f6.dion.ne.jp

H. Aoki
International Apatite Co., Ltd.

the AS or in the modified AS at pH = 7.0, were identified as being octacalcium phosphate (OCP). Decrease in the pH of the surface of the AE and increase in the concentration of phosphate ions were observed by chemical analysis. The OCP precipitation was due to the concentration of phosphate ions and a decrease in the pH on the AE. On the other hands, the precipitation in AS without AE was identified as being a calcium-deficient HA.

2 Material and methods

2.1 Chemical analysis of natural saliva, and preparation of the artificial and modified artificial saliva

Natural saliva samples were collected after a period of fasting from 30 Japanese volunteers (22 men and 8 women) aged between 20 and 30. The inorganic components of the saliva were then analyzed. The sodium, potassium, and chlorine contents were analyzed using an electrode method; the calcium, phosphate, and magnesium contents were analyzed using a spectrophotometer (Hitachi 7450 Autoanalyzer); and the sulfur content was analyzed using a Nippon Dionex DX-AQ1110 chromatograph. Changes in the concentration of the calcium and phosphate ions in the AS were determined using a Varian Techtron Specter AA-250 plus atomic absorption spectrometer, and a Hitachi 150-20 spectrophotometer, respectively. From the analytical mean values of natural saliva samples, the AS was prepared by mixing Na_3PO_4, $NaCl_2$, KCl, $CaCl_2$, $MgCl_2$, H_2SO_4, $NaHCO_3$, and distilled water. A modified meta-stable AS solution with a Ca/P ratio = 0.64 was prepared by adding 1.25 ml of 0.1 M $CaCl_2$ to 100 ml of AS with a Ca/P ratio = 0.28. A modified meta-stable AS solution with a Ca/P ratio = 1.93 was prepared by adding approximately twice the number of calcium ions and one-third the number of phosphate ions of the AS. The pH of the AS and the modified AS were then adjusted using small volume of HCl and KOH containing less than 1% of Cl and K ions in the AS.

2.2 Preparation of artificial enamel

The AE was prepared by sintering HA powder that had been synthesized using a wet method at a temperature of approximately 1,200°C for one hour. The chemical compositions of the AE were analyzed using an Inductively Coupled Plasma Emission Spectrometer (ICP). The AE was identified as being pure polycrystalline HA without incurring any thermal decomposition, and was cut into samples that were 1 cm wide × 1 cm long × 1.5 mm thick using a low-speed saw (Isomet®, Buehler Ltd.). The surface of the samples was polished using # 4000 whetstones. The average weight of the AE was approximately 0.46 g with relative density 97%.

2.3 Precipitation experiments on artificial enamel in artificial saliva

An AE samples weighing 0.46 g were immersed in 100 ml of the AS or modified AS at 37°C at pH = 7.0 for 2 days and 4 weeks. The AE were dried in desiccators at 20°C for 24 h, and the weight changes of the AE were accurately determined using a chemical balance, both before and after rinsing the AE in the AS. The products precipitated on the AE were analyzed without grinding using a Rigaku RINT2200 XRD, a JEOL JSM-5310LVB SEM, and a JEOL EX-5401OMS EDX. The changes of pH on surface of the AE and the AS bulk solution were measured using a 2 mm diameter World Precision Instruments Beetrode® micro pH electrode. Changes of phosphate ion concentration on the AE and the AS bulk solution were measured by the phosphovanadomolybdate method using a spectrophotometer.

2.4 Spontaneous precipitation in modified artificial saliva

Spontaneous precipitation experiments were carried out in a modified artificial saliva having a Ca/P ratio = 0.70 at 37°C, without adding AE samples. The products precipitated in this solution and the products heated at 1,000°C were analyzed by XRD.

Table 1 Compositions of natural and artificial saliva

Elements	Natural saliva in this study* (mM)	Artificial saliva in this study (mM)	Natural saliva in references (mM)
P	3.90 ± 0.61 (3.00∼5.25)	3.90	2.00∼15.40
Na	19.22 ± 8.33 (5.00∼35.00)	19.22	6.00∼32.00
Cl	21.46 ± 5.38 (10.00∼31.00)	24.79	17.00∼100
K	17.89 ± 0.51 (12.50∼22.70)	17.89	14.00∼32.00
Ca	1.10 ± 0.31 (0.65∼1.70)	1.10	1.00∼3.00
Mg	0.08 ± 0.05 (0.04∼0.29)	0.08	0.04∼0.50
CO_3^{2-}	–	3.28	1.63∼30.0
SO_4^{2-}	0.50 ± 0.34 (0.19∼1.02)	0.50	–

*: Mean ± SD (Min ∼ Max)

3 Results

3.1 Chemical analysis of natural saliva, and preparation of the artificial and modified artificial saliva

Compositions of analyzed natural saliva samples, prepared AS samples in this study, and natural saliva in literature references are shown in Table 1. The literature compositions have wide range values of inorganic compositions. The compositions of this study are within the literature range. The chemical reagents used include: $Na_3PO_4 = 3.90$ mM, $NaCl_2 = 4.29$ mM, KCl = 17.98 mM, $CaCl_2 = 1.10$ mM, $MgCl_2 = 0.08$ mM, $H_2SO_4 = 0.50$ mM, $NaHCO_3 = 3.27$ mM, and distilled water. A slight difference in the final chlorine contents from expected values less than 1% was found.

3.2 Preparation of artificial enamel

Figure 1(A) shows the XRD patterns from the surface of AE samples, and these are typical patterns of HA. Fig. 2(A) shows an SEM photograph of the surface in Fig. 1(A). All peaks in the XRD pattern in Fig. 1(A) was identified as pure HA by 9-0432 in the JCPDS. No preferred orientation of the HA crystals was evident from the relative intensities of the each peaks in the XRD patterns. The AE was composed of polycrystals of HA having a Ca/P ratio = 1.66 ± 0.01 and a relative density of 97%. The chemical compositions of the AE were composed of approximately 99.8% of stoichiometric HA and other elements including 1,600 ppm of Mg, 190 ppm of Sr, 180 ppm of Si, 87 ppm of Fe, 28 ppm of Na, 24 ppm of Mn, 16 ppm of K, 8.5 ppm of Cr, less than 5 ppm of Pb, 4.7 ppm of Zn, 3.4 ppm of Ba, 0.9 ppm of Cd, 0.7 ppm of Cu, and 0.6 ppm of Ni.

3.3 Precipitation experiments on artificial enamel in artificial saliva

No precipitation occurred in a solution of AS alone. On the other hand, when the AE was placed into the AS, the weight of the AE sample slowly increased by 0.1–0.3 wt% over 2 days, and by 1.2–1.3 wt% after 4 weeks. This weight increase was considered to be caused by the precipitation of calcium phosphate on the AE. The thickness of the precipitation layer on the AE increased to 1.1–1.6 μm after 2 days, and to 14–15 μm after 4 weeks. The XRD, SEM, and EDX identified the precipitation as being composed of OCP. The pH values of the solution at the surface of the AE samples in the AS decreased from pH = 7.0 to pH = 6.8. On the other hand, the pH value of the AS solution increased by 1.71% ± 0.03 after 1 day, by 1.92% ± 0.03 after 2 days, and by 7.42% ± 0.04 after 1 week. The phosphate ion concentration in the bulk AS decreased by 3.75% ± 0.02 after 1 day, by 4.25% ± 0.03 after 2 days, and by 7.75% ± 0.02 after 1 week. The XRD, SEM, and EDX showed that the precipitation on the AE was always the OCP in the Ca/P ratios of 0.28–1.93 of the AS. The Ca/P ratios of the precipitation were 1.35 to 1.62. The higher Ca/P ration of 1.62 depends on the OCP and HA of the AE. As the small amounts of OCP precipitated in Ca/P ratio of 1.93 XRD and EDX observed the OCP and the surface of the AE. Figures 1(B), (C), and (D) show the XRD patterns of the surface of the AE after soaking at 37°C in an AS with a Ca/P ratio = 0.28 for a period of 4 weeks, in a modified AS with a Ca/P ratio = 0.64 for 2 days, and in a modified AS with a Ca/P ratio = 1.93 for 2 days, respectively. The (100) reflection of OCP at $2\theta = 4.8^\circ$ was observed in these XRD patterns, as

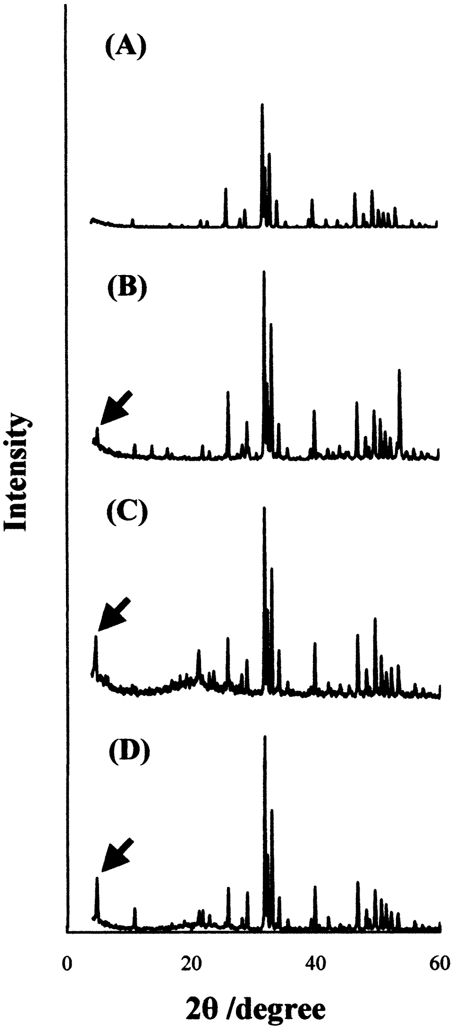

Fig. 1 XRD patterns of products that precipitated on surface of the artificial enamel in artificial and modified artificial saliva at 37°C. Arrows indicated the (100) peaks at $2\theta = 4.8^\circ$ of OCP. (A) Artificial enamel as control with Ca/P = 1.66. (B) After 4 weeks in artificial saliva with Ca/P = 0.28. (C) After 2 days in modified artificial saliva with Ca/P = 0.64. and (D) After 2 days in modified artificial saliva with Ca/P = 1.93

indicated by the arrows. All the XRD patterns were consistent with those of OCP in the JCPDS database (Card # 26-1056). Figures 2(B), (C), and (D) show SEM photographs corresponding to Figs. 1(B), (C), and (D). These patterns are typical of OCP crystals and have a ribbon-like shape. The Ca/P ratios of these precipitates were in the range 1.35–1.62. As the precipitation thickness in Ca/P 1.62 is approximately 1.0 μm, the Ca/P values include both OCP and HA of substrate.

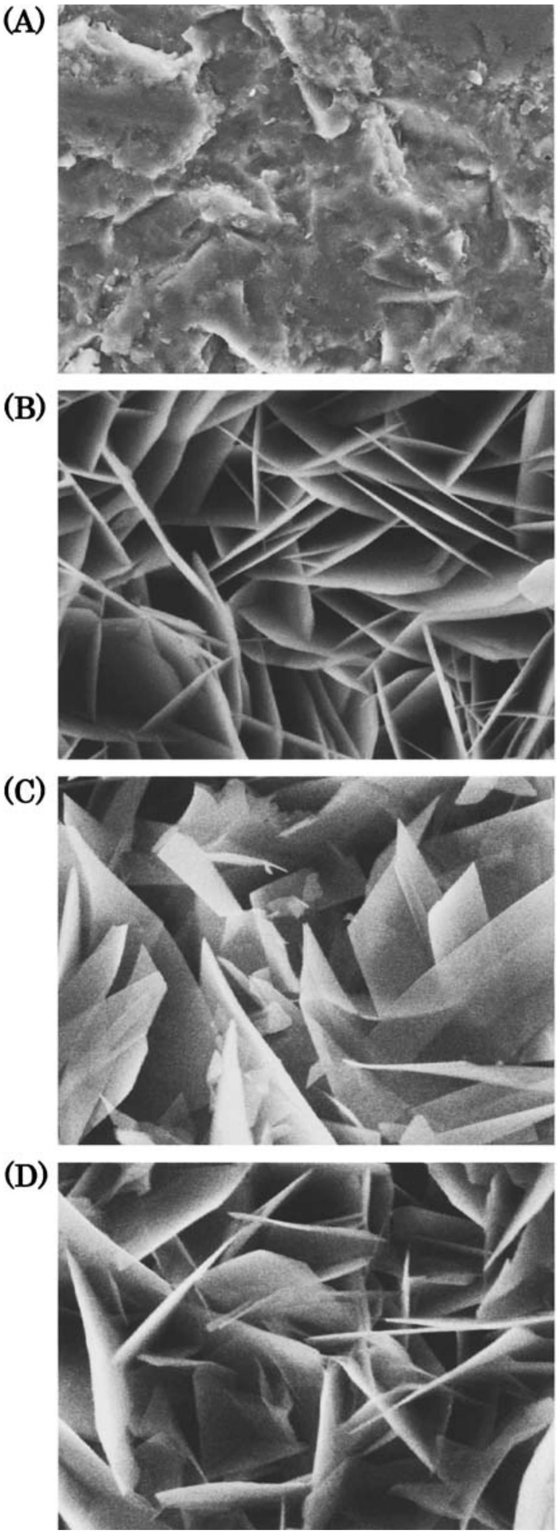

Fig. 2 SEM photos of products that precipitated on surface of artificial enamel in artificial and modified artificial saliva at 37°C corresponding to the XRD patterns in Fig. 1(A), (B), (C) and (D). (A) Artificial enamel as control with Ca/P = 1.66 (×2000). (B) After 4 weeks in artificial saliva with Ca/P = 0.28 (×7500). (C) After 2 days in modified artificial saliva with Ca/P = 0.64 (×7500). and (D) After 2 days in modified artificial saliva with Ca/P = 1.93 (×7500)

3.4 Spontaneous precipitation in modified artificial saliva

Spontaneous precipitation occurred in the modified AS bulk solution having a Ca/P ratio = 0.70 at 37°C without adding AE. The precipitation was identified as being HA from the XRD data shown in Fig. 3(A). The all XRD patterns belong to low crystalline HA, and the Ca/P ratio was determined to be 1.58 ± 0.02. Figure 3(B) shows the XRD pattern obtained after heating the precipitation at 1,000°C for 1 h. Referencing this pattern to JCPDS database card number 9-0169 showed that the data in Fig. 3(B) represented a mixture of β-tricalcium phosphate (β-TCP) and HA. From these results it was concluded that the precipitation was identified as a calcium-deficient hydroxyapatite, which is amorphous calcium phosphate (ACP).

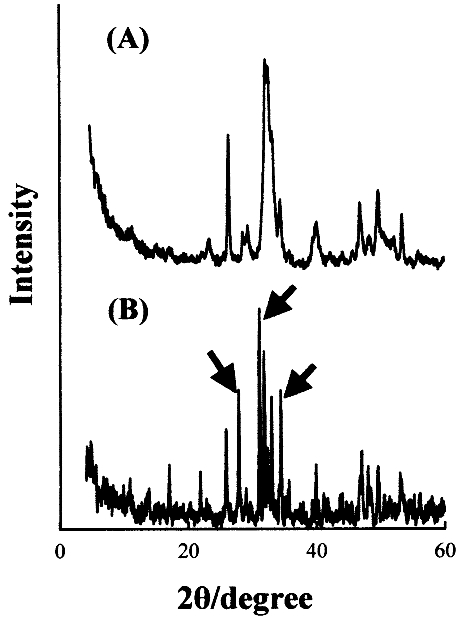

Fig. 3 XRD pattrens of products that precipitated in the bulk of modified artificial saliva with Ca/P = 0.70 at 37°C. Arrows indicated β-TCP peaks. (A) Before heating. and (B) After heating at 1000°C for 1 h (HA and β-TCP)

4 Discussion

The AS possessed a calcium/phosphate molar ratio of 0.28, and had a pH = 7.0, which reflected the values from the natural saliva samples, and of literature references. The AE was composed of HA polycrystals with a relative density of 97 %, and a Ca/P ratio = 1.66 ± 0.01. The HA polycrystals had no preferred orientation, and were approximately 1–2 μm long, while natural enamel is composed of smaller HA crystals with sizes of 0.1–1.0 μm that are orientated along the crystallographic *c*-axis of HA. Little difference in chemical property will be considered between the AE and natural enamel.

Various calcium phosphates are known to precipitate in solutions containing calcium and phosphate ions in the pH range, $1.0 < \text{pH} < 7.0$ [9]. Octacalcium phosphate with a Ca/P ratio = 1.33 will be precipitated in solutions with a pH = 5.0–6.0. In 1958, Trautz et al. reported that OCP precipitated as ribbon-like crystals on enamel in a solution at pH = 5.0–6.0 at 40°C [10]. However, in this study the OCP always precipitated on the AE in an AS with pH = 7.0 and Ca/P ratios of 0.28, 0.64, and 1.93 at 37°C. Decrease in the pH of the surface of the AE and increase in the concentration of phosphate ions were observed by chemical analysis. The OCP precipiation was due to the concentration of phosphate ions and a decrease in the pH on the AE.

On the other hand, when spontaneous precipitation occurred in the modified AS with a Ca/P ratio = 0.70 without adding the AE, the product was a calcium deficient HA even though the Ca/P ratio of the modified AS was 0.70, which was lower than the Ca/P ratio of HA of 1.67. The calcium-deficient HA will decompose into β-tricalcium phosphate (β-TCP) and HA at 1,000°C [11–13]. The Ca/P ratio of the precipitate was 1.58 ± 0.02, then the chemical formula of the calcium deficient hydroxyapatite was determined as $Ca_{9.48}(PO_4)_6(OH)$ by EDX analysis.

From these results, it was concluded that OCP precipitates on the AE in the AS by concentrating the phosphate ions and decreasing the pH. From these results, it was concluded that OCP precipitates on the AE in the AS by concentrating the phosphate ions and decreasing the pH. On the other hands, in 1957 Brown WE et al. reported that at the early stage of calculus generation in oral cavity brushite ($CaHPO_4 \cdot 2H_2O$) will deposit in pH ranges of 3.0–4.0, then the brushite slowly hydrolyses to OCP [14]. Furthermore the OCP changes into hydroxyapatite. There are many factors to decrease pH in actual oral cavity including bacteria, foods, enzymes, and local structure between teeth. In the present study their factors except AE and AS does not include. Therefore, the pH on the AE in AS does not decreases to 3.0–4.0 to deposit brushite.

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