INFLUENCE OF STRONTIUM(II) ON THE FORMATION OF CALCIUM HYDROXYAPATITE PREPARED BY HYDROLYZING PHENYL PHOSPHATE USING ALKALINE PHOSPHATASE AT 37°C

Hidekazu Tanaka*, Suguru Nishimoto
(*Corresponding author: hidekazu@riko.shimane-u.ac.jp)

Department of Material Science, Faculty of Science and Engineering, Shimane University, 1060 Nishikawatsu, Matsue, Shimane 690-8504, Japan

Keywords: Calcium hydroxyapatite, Strontium, Phenyl phosphate, Alkaline phosphatase, Body temperature

Abstract: Influence of Sr²⁺ on the formation and structure of calcium hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂: CaHap) particles prepared by hydrolyzing the phenyl phosphate (C₆H₅PO₄H₂) using alkaline phosphatase (ALP) at 37°C was examined. Increasing the additional amount of Sr²⁺ increased the Sr content in the products and turned the crystal phase of the products as follow: CaHap → solid solution SrCaHap → Sr₁.7Ca₁.3(PO₄)₂ → (3Sr(HPO₄)₂•Sr₃(PO₄)₂)•2H₂O. All the phenyl phosphates were hydrolyzed by ALP at 37°C. The crystallinity and particle size of CaHap decreased with an increase of added Sr²⁺, meaning the inhibition of crystallization and particle growth. These facts suppose that incorporation of Sr²⁺ in animal organism suppresses the formation of hard tissues.

(Received June 19, 2011; Accepted August 11, 2011)

INTRODUCTION

Calcium phosphate and organic phosphate are of great importance in animal organisms. Calcium hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂: abbreviated as CaHap) is a major component of teeth and bones [1]. While, organic phosphates form cell wall, nucleosome and ATP (adenosine triphosphate) in a human body, and a part of organic phosphates are hydrolyzed to form inorganic phosphate by enzymes such as alkaline phosphatase (ALP) and acid phosphatase (ACP). Anderson proposed the formation mechanism of CaHap in vivo as follows: (1) hydrolysis of organic phosphates to form PO₄³⁻ ions and (2) reaction of Ca²⁺, PO₄³⁻ and OH⁻ ions to generate CaHap [2]. In order to elucidate more detail of this mechanism, information about interaction between organic phosphate and CaHap is required. There are many studies reported about the interaction by Bottari and Porto [3], Suga and Okabe [4], Lebugle and co-workers [5–7], Ozin et al. [8], Kandori et al. [9] and our group [10–12]. However, influence of hydrolysis of organic phosphates on the formation of CaHap has not been fully discussed except for our studies [13,14]. Clarifying this subject would give us a useful information about formation mechanism of hard tissues in vivo. To elucidate this, simple organic phosphates such as phenyl phosphate (C₆H₅PO₄H₂: PP) are adequate because it is easily hydrolyzed in aqueous media by controlling the solution pH [15,16]. Recently, the author reported the synthesis of CaHap by hydrolyzing the PP in aqueous Ca(OH)₂ solution at 85°C and at body temperature of 37°C in the presence of ALP [13,14].

On the other hand, CaHap can incorporate various cations such as Mg²⁺, Sr²⁺, Ba²⁺, Al³⁺, Cr³⁺, Fe³⁺ and so forth by substituting with Ca²⁺ in the crystal [17–22]. Among them, ⁹⁰Sr is a long-lifetime radioactive element and is easily dissolved in water. Most recently, dumping of radioactive waste water including ⁹⁰Sr, ¹³¹I and ¹³⁷Cs into the sea from the Fukushima nuclear power station damaged by the 2011 off the pacific coast of Tohoku earthquake in Japan is a serious problem. However, in our knowledge, there is no study about preparation of CaHap in the presence of Sr²⁺ using organic phosphate and enzyme at body temperature.

The aim of this study was elucidate the influence of Sr²⁺ on the formation and structure of CaHap at body temperature. So that, the CaHap particles were prepared by hydrolyzing the PP with alkaline phosphatase ALP in aqueous media containing Ca²⁺ and Sr²⁺ at 37°C. The products formed were characterized by various means.

MATERIALS AND METHODS

Materials

The samples were prepared as follows. Ca(OH)₂ and Sr(OH)₂ were dissolved into 450 ml of deionized-distilled water free from CO₂ in a sealed polypropylene vessel. Then, the total amount of Ca and Sr was 8.85 mmol and the atomic ratio Sr/(Ca+Sr) ranged from 0 to 1.0. Hereafter,
Sr/(Ca+Sr) ratio in starting solution is abbreviated as XSr. To the solution were added 50.0 ml of 0.106 mmol/dm³ aqueous PP solution. After this, aqueous solution (10 ml) including 10 units of ALP (Biozyme Laboratories, source: bovine intestinal mucosa, specific activity: 1.5 units/mg of enzyme, 1 unit is defined as the quantity of enzyme which catalyses the reaction of 1 μmol of substrate per minute) was added to the solution. Then, the solution pH was adjusted to 9.6 by adding 1.0 mol/dm³ aqueous HNO₃ solutions and the suspension was aged at 37°C for 48 h in an incubator. After aging, the product generated was filtered off, thoroughly washed with deionized-distilled water and finally dried at 50°C in an air oven for 24 h.

Characterization

The materials thus obtained were characterized by a variety of conventional techniques. Powder X-ray diffraction (XRD) patterns were taken on a Rigaku RINT-2100 diffractometer with a Ni-filtered CuKα radiation (30 kV, 16 mA). Particle morphology was observed by a TOPCON EM-002B transmission electron microscope (TEM). Ca and Sr contents were assayed by a Shimadzu A-6200 atomic absorption spectroscopy (AAS). The samples were dissolved in 10 mol/dm³ aqueous HCl solution. Carbon content was determined by a Yanagimoto MT-6 CHN elemental analyzer. IR spectra were recorded by a KBr method using a JASCO FTIR 660 plus Fourier transform infrared (FT-IR) spectroscopy.

RESULTS AND DISCUSSION

Figure 1 shows the XRD patterns of the products formed at different XSr. Pattern a of the product obtained at XSr = 0 shows diffraction peaks at 2θ = 25.8°, 28.2°, 28.8° and 31.8°. These peaks can be assigned to the CaHap (JCPDS no. 9-432). The formation of CaHap by hydrolyzing the PP using ALP at 37°C was reported by following reactions [14]:

\[ \text{ALP} + \text{C}_6\text{H}_5\text{PO}_4^{2-} \rightarrow \text{ALP-C}_6\text{H}_5\text{PO}_4^{2-} \]  
\[ \text{ALP-C}_6\text{H}_5\text{PO}_4^{2-} + \text{H}_2\text{O} \rightarrow \text{PO}_4^{3-} + \text{C}_6\text{H}_5\text{O}^- + \text{H}^+ + \text{ALP} \]  
\[ 10\text{Ca}^{2+} + 6\text{PO}_4^{3-} + 2\text{OH}^- \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \]  
\text{(CaHap)} (3)

As XSr increases up to 0.60, the diffraction intensity of CaHap peaks weakens and the peaks shift to a lower diffraction angle (patterns a - d). Similar result was reported by Ishikawa et al., who synthesized the solid solution strontium-calcium hydroxyapatite (SrCaHap) by a mixture of Ca(OH)₂, Sr(OH)₂ and H₃PO₄ in aqueous media [18]. It seems, therefore, that the materials formed at XSr ≤ 0.60 are identified as SrCaHap. This will be discussed later. At XSr = 0.80, the CaHap peaks disappear and new peaks are detected at 2θ = 16.4°, 24.8°, 25.6°, 26.8°, 29.8° and 33.2°, which can be ascribed to the Sr₃(PO₄)₂ (no. 80-1614) (pattern e). Further, the Sr₃(PO₄)₂ peaks vanish and the peaks due to (3Sr(HPO₄)₂·Sr₃(PO₄)₂·2H₂O are recognized at XSr = 1.0 (no. 19-1287) (pattern f). Comparing to the literature values reported by McConell, it can be regarded that the product has enlarged a-direction. This would be due to the substitution of H₂O with OH⁻ in CaHap crystal [21]. The lattice parameters a and c almost linearly increase on increasing XSr. It is known that the CaHap crystal is hexagonal with space group P6₃/m and possesses two kinds of cation sites of cation(I) and cation(II) [24]. The cation(I) and cation(II) sites are columnar and triangular sites, respectively. The large cations preferentially occupy the cation(II) sites while the smaller ones prefer the cation(I) sites. Since the ionic radius of Sr²⁺ (0.118 nm) is larger than that of Ca²⁺ (0.099 nm), the Sr²⁺ is incorporated in cation(II) site in CaHap crystal to form solid solution SrCaHap. However, the a and c values are less than those calculated from XSr represented by dotted lines in Fig. 2, meaning that incorporation of Sr²⁺ in CaHap crystal is difficult.
Figure 3 plots the atomic ratio Sr/(Ca+Sr) of the product calculated from Ca and Sr contents assayed by AAS as a function of XSr. The Sr/(Ca+Sr) ratio in the products slightly enhances on increasing XSr up to 0.60 and then suddenly raises. Nonetheless, the Sr/(Ca+Sr) ratio is much lower than XSr. This can be explained by that the stability constant of PO₄³⁻-complex with Ca²⁺ (1.70) is larger than that with Sr²⁺ (1.52) [25], that is, former complex is more stable than latter one. Also, the phenyl group content represented by open circles in Fig. 3 is negligibly small over the whole XSr range, revealing that all of the PP in the starting solution are hydrolyzed by ALP. At XSr = 0.80, the sample is identified as Sr₃(PO₄)₂ and contains Ca and Sr, inferring that a part of Sr²⁺ is replaced with Ca²⁺. Assuming that chemical formula of this material is Sr₃₋ₓCaₓ(PO₄)₂, the x value estimated from Ca and Sr contents is 1.3.

The crystallite size evaluated from the full width at half height of (002) peak of CaHap and SrCaHap using the Scherrer equation is plotted against XSr by open squares in Fig. 4. Note that the crystallite size is decreased by increasing XSr, implying that incorporation of Sr²⁺ in CaHap crystal inhibits the crystallization of this material. This seems that the ionic radius of Sr²⁺ is larger than that of Ca²⁺.

Figure 5 shows the IR spectra of PP and the products obtained at various XSr. Spectrum a of the PP has absorption bands at 1595, 1491, 1445, 1223, 1101, 978, 768 and 686 cm⁻¹. The 1595-, 1491- and 1445-cm⁻¹ bands are assigned to the ring stretching vibration mode of phenyl groups, 1223-, 1101- and 1032- and 978-cm⁻¹ ones are to the P-O stretching vibration of P-O-C groups, and 768- and 686-cm⁻¹ ones are respectively to the H-C-H and C-C-C deformation vibration modes of phenyl groups. At XSr = 0, the PP bands vanish and new ones develop at 1100, 1033, 599 and 563 cm⁻¹ (spectrum b). The former two bands are ascribed to the O-P-O deformation vibration modes of PO₄³⁻. This finding also implies that all of the PP are

FIGURE 2 Plots of lattice parameters (□) a and (○) c of the CaHap and SrCaHap against XSr.

FIGURE 3 Plots of (□) atomic ratio Sr/(Ca+Sr) in the products and (○) phenyl group content as a function of XSr.

FIGURE 4 Plots of crystallite size of (□) CaHap and SrCaHap, and (○) particle length against XSr.

FIGURE 5 IR spectra of the products formed at different XSr. X₅Sr: (a) 0, (b) 0.20, (c) 0.40, (d) 0.60, (e) 0.80, (f) 1.0.
hydrolyzed during preparation to generate PO$_4^{3-}$ via reactions (1) and (2). No remarkable difference in IR spectra is recognized on increasing XSr up to 0.60 (spectra b - d). At XSr = 0.80, the P-O stretching bands at 1075 and 1028 cm$^{-1}$ and O-P-O deformation ones at 595 and 545 cm$^{-1}$ due to Sr$_{1.7}$Ca$_{1.3}$(PO$_4$)$_2$ appear. In case of XSr = 1.0, the O-H stretching band of P-OH groups of (3Sr(HPO$_4$)$_2$•Sr$_3$(PO$_4$)$_2$)•2H$_2$O is developed at 993 cm$^{-1}$.

Figure 6 shows the TEM pictures of the products formed at different XSr. At XSr = 0, the rod-shaped CaHap particles with the size of ca. 185 nm in length and ca. 18 nm in width are seen in picture a. It has been reported by the author that the CaHap synthesized by hydrolyzing the PP at 85°C is micro-meter sized needle-like particles [13]. The formation of needle-like particles was explained by the gradual hydrolysis of PP and the Ostwald ripening. Whilst, hydrolysis of the PP using ALP is taken place in a short time, leading to the formation of nano-sized CaHap [14]. By incorporation of Sr$^{2+}$, the size of rod-shaped particles is seemed to be decreased. To clarify more detail of this, the particle length of CaHap and SrCaHap is plotted by open circles in Fig. 4. As can be seen, the particle length reduces on increasing XSr. This means the inhibition of particle growth by incorporation of Sr$^{2+}$. Further, the particle length is larger than crystallite size, indicating the products to be polycrystalline. The irregular particles with a size of ca. 2.4 μm are generated at XSr = 0.60 in addition to the SrCaHap ones (picture d). The irregular particles would be assigned to amorphous calcium-strontium phosphates. The irregular particles due to Sr$_{1.7}$Ca$_{1.3}$(PO$_4$)$_2$ are formed at XSr = 0.80 (picture e). Further, the (3Sr(HPO$_4$)$_2$•Sr$_3$(PO$_4$)$_2$)•2H$_2$O particles with a size of ca. 2 μm are developed at XSr = 1.0.

CONCLUSIONS

The results obtained in the present study can be summarized as follows. Addition of Sr$^{2+}$ strongly inhibits the formation, crystallization and particle growth of CaHap synthesized by hydrolyzing the PP using ALP at 37°C. All the PP are hydrolyzed by ALP during preparation. Increasing XSr turned the crystal phase of the products as follow: CaHap → solid solution SrCaHap → Sr$_{1.7}$Ca$_{1.3}$(PO$_4$)$_2$ → (3Sr(HPO$_4$)$_2$•Sr$_3$(PO$_4$)$_2$)•2H$_2$O. This reveals that higher XSr suppresses the formation of CaHap.

These facts suggest that incorporation of Sr$^{2+}$ in animal organism suppresses the formation of hard tissues, which may cause the depression of mechanical strength of teeth and bones.

ACKNOWLEDGEMENTS

The authors thank to Mr. Tsunao Yoneyama of Department of Biosignaling and Radioisotope Experiment Center for Integrated Research in Science of Shimane University for his help with TEM observation.

REFERENCES


FIGURE 6 TEM pictures of the products formed at different XSr. XSr: (a) 0, (b) 0.20, (c) 0.40, (d) 0.60, (e) 0.80, (f) 1.0.