

Analysis of Inorganic Substance Deposited *In vivo*

Giichiro Kawachi, Yoji Tamura*, Hirotaka Fujimori*, Seishi Goto* and Koji Ioku

Graduate School of Environmental Studies, Tohoku University
Aoba 20, Sendai, Miyagi 980-8579, Japan

Fax: + 81-22-217-7407, e-mail: ioku@mail.kankyo.tohoku.ac.jp

*Applied Medical Engineering Science, Graduate School of Medicine, Yamaguchi University,
2-16-1 Tokiwadai, Ube, Yamaguchi 755-8611, Japan

The inorganic matter of bladder stones and calcinoma deposited morbidly in the human muscle was characterized by instrumental analysis. The chemical analysis was precisely carried out by AAS, ICP-AES and ICP-MS. Bladder stones contained inorganic matter of about 95% and organic matter of about 5%. According to XRD and FT-IR, the inorganic matter of bladder stone was about 60% apatite and about 40% calcium oxalate. From the chemical analysis, Ca/P molar ratio as apatite was 1.72. In the contrary, the calcinoma contained inorganic matter of about 40% and organic matter of about 60%. The amount of inorganic matter was less than that of natural bones. According to XRD and FT-IR, the inorganic matter was carbonateapatite. Carbonate ion partially existed in PO₄ site and OH site in apatite structure. OH ion was appeared after heating above 500 °C. From ¹H NMR, OH ion was appeared under 500 °C. Elements other than Ca and P were detected from the chemical analysis, and Ca/P molar ratio of it was 1.98. It was suggested that the chemical composition of the apatite in calcinoma was as follows. [Ca_{7.61}Mg_{0.16}Na_{0.29}X_n][(PO₄)_{3.84}(CO₃)_{2.16}Y_m][CO₃]_{0.32}(OH)_{0.34}(H₂O)_{1.34}], where X=Al, K, Zn, Fe, Sr with n<0.01, and Y=SiO₄, SO₄ with m<0.01.

Key words; apatite, bladder stone, calcification, chemical analysis

1. INTRODUCTION

The pathological inorganic crystal deposits are sometimes observed in muscles as the ectopic calcification. The normal calcification in bones and teeth is well known as calcium hydroxyapatite formation and it was biologically controlled. It has been established that ectopic calcification is apatite deposition as well. However, ectopic calcification has not been precisely investigated in detail. Although a large number of studies have been made on urinary calculus as elementally analysis, little is known as apatite structure. The authors believe that apatite is one of the key material for life science and engineering, therefore natural apatite should be characterized.

The measurement of trace and major elemental components of human biological material is important in the detection of disease and in the investigation of poisoning. Inorganic substances deposited *in vivo* have a complicated composition and knowledge of its composition is of fundamental importance in the application and interpretation of elemental analysis.

In this study, bladder stones and calcinoma deposited pathologically in muscles were focused only on inorganic matters and the samples were analyzed precisely. The analysis is not only for elucidation of some disease but also for designing and preparation of advanced biomaterials like a scaffold of tissue engineering for cultured bones [1, 2].

2. EXPERIMENTAL PROCEDURE

2.1 Bladder stones

The sample was removed from 26-year-old male who was diagnosed as a vesicolithiasis. The sample was washed de-ionized water and ethanol. Then it was dried at 100 °C for 24 h in air.

2.2 Calcinoma

The sample as phyma in the muscle of buttocks was removed from 68-year-old female who was diagnosed as a CREST (calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly and telangiectasia) syndrome. In order to eliminate organic substance, the sample was ground and then soaked in aqueous ammonia with pH=10 at the room temperature for 12 h. After this treatment, it was dried at 100 °C for 24 h in air.

2.3 Analysis

The sample were characterized by optical microscopy, scanning electron microscopy (SEM; JEOL JSM-25S, Japan), X-ray diffractometry (XRD; Rigaku Geiger flex, Japan) and thermal analysis (TG-DTA; Seiko Instruments Inc. TG/DTA32, Japan).

FT-IR spectra were obtained by KBr method using Fourier transform infrared spectroscopy (FT-IR; Perkin Elmer Spectrum 2000, USA). The sample was diluted with spectroscopic grade KBr and about its concentration was 2 mass%. Measurements were taken in N₂ at room

temperature. Spectral resolution was 4cm^{-1} and the number of scans was 10.

Chemical composition was measured for the sample after heating at $900\text{ }^\circ\text{C}$ (Calcinoma) or $500\text{ }^\circ\text{C}$ (Bladder stones) by atomic absorption spectrometry (AAS; Hitachi Z-5310, Japan), inductively coupled plasma atomic emission spectrometry (ICP-AES; SPS7800 Seiko Instruments, Japan) and inductively coupled plasma mass spectrometry (ICP-MS; SPQ9000 Seiko Instruments, Japan).

Raman measurements for samples have been performed by using the system that is based on a UV argon-ion laser (363.8nm), a spatial filter, a single monochromator couples to a double-grating rejection filter, and a two-dimensional charge-coupled device (CCD) detector. Its detail was described in the past report [3].

The NMR analysis was performed on a spectrometer (CMX-300 Chemagnetics, Div. of Varian, Inc., Palo Alto, CA), using 11 kHz MAS . The resonance frequency used was 300.7 MHz for ^1H . A standard 4 mm "oencil" model probe (Chemagnetics) was used with ZrO_2 rotor. The magic angle was set before insertion of the sample, by optimizing the response of the ^{79}Br signal of the spinning sidebands of KBr (Maciel's method) [4]. Pulse length was calibrated before analysis, using silicone rubber (poly-dimethylsiloxane). A single-pulse sequence was used for measuring ^1H , as OH, in samples. The chemical shifts were referenced to silicone rubber (poly-dimethylsiloxane), whose resonance was checked by inserting it into an MAS rotor (0.119 ppm , $>2\text{ kHz MAS}$) [5]. All freeinduction decays were subjected to standard Fourier transformation and phasing.

3. RESULTS AND DISCUSSION

3.1 Bladder stones

The removed bladder stones was shown in Fig. 1. Two shape crystal were observed. Platy shape was whewellite ($\text{Ca}(\text{COO})_2 \cdot \text{H}_2\text{O}$) and rhombohedron one was weddellite ($\text{Ca}(\text{COO})_2 \cdot 2\text{H}_2\text{O}$) [6]. The structure and chemical composition of bladder stones is non-homogeneous [7]. The content of organic substances in the sample was measured by thermal analysis.

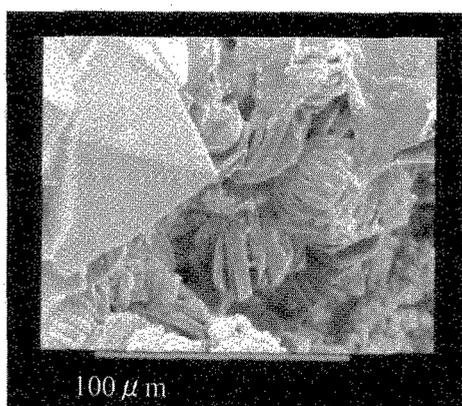


Fig. 1 SEM image of bladder stones.

According to TG-DTA (Fig. 2), there was three stages as weight loss. At the temperatures from about $150\text{ }^\circ\text{C}$ to about $250\text{ }^\circ\text{C}$ due to evaporation of hydrous water, from about $250\text{ }^\circ\text{C}$ to about $400\text{ }^\circ\text{C}$ due to burning of organic substances, around $480\text{ }^\circ\text{C}$ was decomposition of calcium oxalate of $\text{Ca}(\text{COO})_2$ to form CaCO_3 , and around $700\text{ }^\circ\text{C}$ was decomposition of calcium carbonate to form CaO . The second stage of weight loss means the loss of organic substances, therefore there were about 5% organic and about 95% inorganic substances in this sample.

The patterns of XRD for the samples showed that whewellite, weddellite and apatite were detected after drying at $100\text{ }^\circ\text{C}$ and calcite and apatite were detected in the sample after heating at $500\text{ }^\circ\text{C}$ 1h in air (Fig. 3). There was no CaCO_3 heated at $100\text{ }^\circ\text{C}$. So CaCO_3 in the sample heated at $500\text{ }^\circ\text{C}$ was regarded as pyrolysate of calcium oxalate. According to quantitative analysis from XRD, apatite concentration in bladder stones was about 60% . This result was corresponding with that of TG results.

According to IR spectra of the sample after drying at $100\text{ }^\circ\text{C}$, characteristic bands of organic substances weren't recognized (Fig. 4). The sample had CO_3^{2-} and little OH^- in the apatite structure. After heating at the temperatures above $500\text{ }^\circ\text{C}$, OH^- was clearly found in apatite structure because of de-carbonation and substitution of OH^- for CO_3^{2-} in OH site of apatite.

After heating at $500\text{ }^\circ\text{C}$ 1h in air, the chemical analysis of the sample was carried out. The elements in this sample were shown in Table 1. According to quantitative analysis, apatite concentration of bladder stones was about 60% . So, the Ca/P molar ratio of apatite in bladder stones was 1.72 , which was quite larger than the value of stoichiometric apatite of 1.67 . The reason that Ca/P molar ratio of this sample was larger than that of stoichiometric value was substitution of CO_3^{2-} for the PO_4 site in apatite, therefore the amount of P was smaller than that of stoichiometric apatite.

Raman spectroscopic analysis was used for investigation of existence of OH^- ion in apatite structure. But well-defined spectrum wasn't obtained because of containing organic substances.

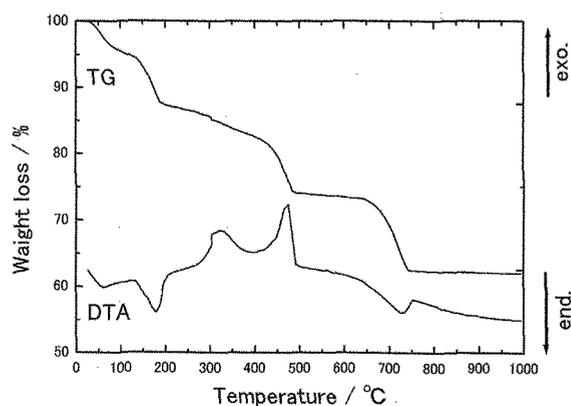


Fig. 2 TG and DTA curves of bladder stones.

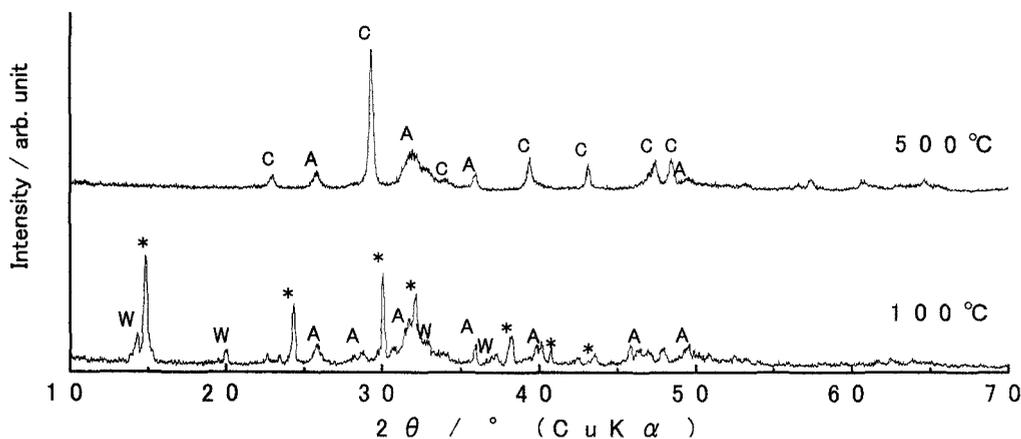


Fig. 3 Patterns of XRD of bladder stones after heating at 100 °C and 500 °C.

A; Hydroxyapatite, C; Calcium carbonate, *; weddellite, w; whewellite

Table 1 Chemical composition of inorganic substances in bladder stones analyzed by AAS, ICP-AES and ICP-MS.

Ca	Mg	Na	K	Sr	Zn	Fe	Al	P	Si	S
mass %										
37.3	0.46	0.55	0.14	0.02	0.08	0.004	0.01	9.81	0.006	0.06

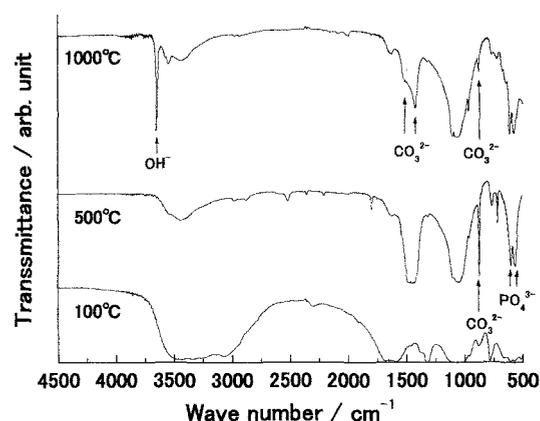
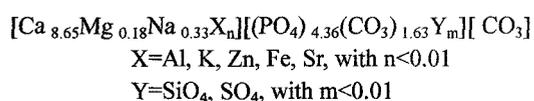


Fig. 4 FT-IR spectra of bladder stones after heating at indicating temperatures.

3.2 Calcinoma

In the past paper, we reported the chemical analysis of calcinoma [8]. The calcinoma contained inorganic matter of about 40 % and organic matter of about 60 %. The amount of inorganic matter was less than that of natural bones. According to XRD and FT-IR, the inorganic matter was carbonate apatite. Carbonate ion partially existed in PO₄ site and OH site in apatite structure. OH ion was appeared after heating above 500 °C. Elements other than Ca and P were detected from the chemical analysis, and Ca/P molar ratio of it was 1.98, which is over the stoichiometric value of 1.67. The formula of apatite on the present study was decided based on the formula previously reported [9]. According to FT-IR, it seemed that there was no OH⁻ in the apatite structure. The chemical composition of the apatite in calcinoma was as follows.



But it is hard to deposit full content carbonate apatite in aqueous system. From FT-IR, OH ion in apatite structure appeared after heating above 500 °C in air. In case of bone mineral, OH ion appeared after heating above 550 °C in air and OH site was vacancy or substitution of H₂O [10]. IR can provide information about the hydroxyl group in hydroxyapatite. However, reliable quantitation is difficult, particularly for the other hydrogen-containing species. Raman was more sensitive than IR. And the inherently quantitative nature of the NMR renders it an attractive technique for the structural investigation of apatite. In typical ¹H NMR spectrum of hydroxyapatite, 0ppm is hydroxyl group, 5ppm is lattice water and 8ppm is surface absorbed water [11].

Raman and ¹H NMR were used for investigation of existence of OH ion in apatite structure. In order to determine the existence of OH in apatite structure under 400 °C, the samples were heated at 250 °C, 400 °C and 900 °C in air. Raman was failed. Well-defined spectrum wasn't obtained because of containing organic substances. 0ppm appeared in all samples (Fig. 5). It was suggested that apatite in calcinoma had OH ion in its structure. The spectrums changed by heating, but 0ppm and 5ppm were almost same between 250 °C and 400 °C. It was suggested

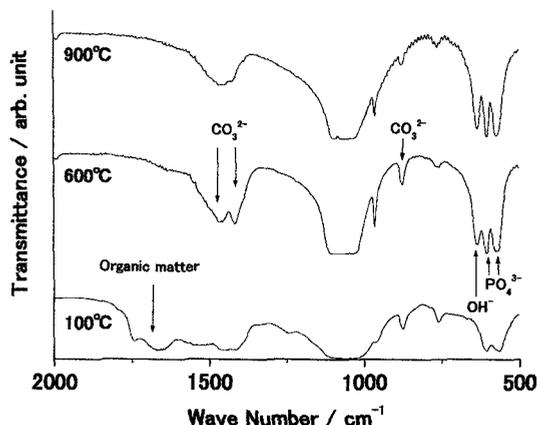
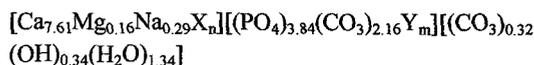


Fig. 4 FT-IR spectra of calcinoma after heating at indicating temperatures.

that OH ion and lattice water didn't change by heating from 250 °C to 400 °C. In NMR analysis, there is correlation between amounts of proton and its intensity, integral intensity can be regarded as amount of proton. Amount of OH ion after heating 400 °C was about 0.6 times smaller than that of 900 °C. This value was smaller than synthetic stoichiometric hydroxyapatite (Ca/P=1.67). After heating 400 °C, the proton as lattice water was as same as OH ion.

Carbonate ion partially existed in PO₄ site (type B) and OH site (type A) in apatite structure [12]. Carbonate ion in structure of apatite was analyzed quantitatively with calcium carbonate (875cm⁻¹) [13]. PO₄ site was about 36% substitution of CO₃²⁻ and OH site was about 16%. The rest of OH site was regarded as substitution of lattice water and OH ion. The structural formula of apatite in calcinoma could be supposed as follows.



X=Al, K, Zn, Fe, Sr with n<0.01

Y=SiO₄, SO₄ with m<0.01.

4. CONCLUSION

The inorganic substance in bladder stones contained carbonate apatite and whewellite (Ca(COO)₂ · H₂O), weddellite (Ca(COO)₂ · 2H₂O).

The inorganic substance in calcinoma was carbonate apatite. It contained OH ion in its structure. According to the chemical analysis and solid state characterization, its structural formula could be clarified.

Acknowledgements

Calcinoma was provided from department of orthopedic surgery, University of Tokyo. Bladder stones was provided from urology, the National Kyushu Medical Center.

The authors are thankful to Prof. M. Yoshimura for his

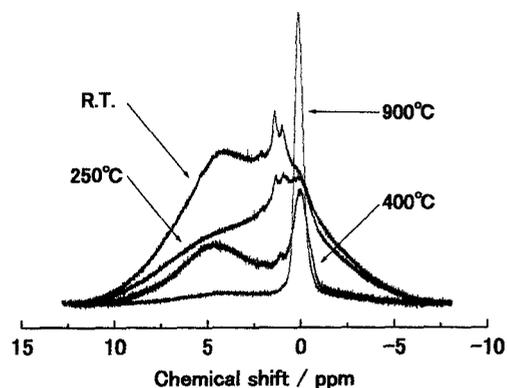


Fig. 5 ¹H NMR spectra of calcinoma after heating at indicating temperatures.

help in UV Raman system for measurement.

Finally the authors wish to thank Mr. M. Fujimura of Konoshima Chemical Industry Co. Ltd. for his help of chemical analysis, and also wish to thank Dr. Y. Suetsugu of the National Institute for Materials Science for his useful suggestions about crystal structure of apatite.

REFERENCES

- [1] R. Langer and J. P. Vacanti, *Science*, **260**, 920-926 (1993).
- [2] K. Ioku, *Chem. Ind.*, **52** [5], 360-365 (2001).
- [3] M. Yashima, M. Kakihana, R. Shimidzu, H. Fujimori and M. Yoshimura, *Appl. Spectrosc.*, **51**, 1224 (1997)
- [4] J. S. Frye and G. E. Maciel, *J. Magn. Reson.*, **48**, 125-131 (1982)
- [5] S. Hahashi and K. Hayamizu, *Bull. Chem. Soc. Jpn.*, **64**, 685-687 (1991)
- [6] H. Takeuchi and O. Yoshida, *Zin to toseki (Kidney and dialysis)*, **23**, 456-461 (1987).
- [7] T. Koide and T. Sonoda, *Zin to toseki (Kidney and dialysis)*, **23**, 473-478 (1987).
- [8] K. Ioku, G. Kawachi, H. Fujimori, S. Goto, K. Fujiwara, M. Waranabe, H. Oda, S. Tanaka, T. Matsumoto, *Trans. Mater. Res. Soc. Japan*, **27** [2], 455-457 (2002).
- [9] Y. Suetsugu, *Rigaku-Dennki J.*, **27** [2], 12 (1996).
- [10] R. M. Biltz and E. D. Pellegrino, *J. Dent. Res.*, **62** (12), 1190-1195 (1983).
- [11] James P. Yesirowski and Hellmut Eckert, *J. Am. Chem. Soc.*, **109**, 6274-6282 (1987)
- [12] Y. Suetsugu, *J. Soc. Inorg. Mater. Japan*, **3**, 48-54 (1996).
- [13] K. Ioku, M. Yoshimura and S. Somiya, *Nippon Kagaku Kaishi (J. Chem. Soc. Japan)*, **9**, 1565-1570 (1988).