INTRODUCTION

Hydroxyapatite (Ca_{10}(PO_{4})_{6}(OH)_{2}; HAp) is a biocompatible and osteoconductive compound, which is a major inorganic component of living hard tissue. However, synthesized HAp is typically not suitable for use in autogenous bone grafts because of its unsatisfactory clinical outcomes. Therefore, development of HAp with better performance is necessary. Compared to synthetic HAp, biological apatite contains a variety of inorganic ions including sodium (Na\(^+\)), magnesium (Mg\(^{2+}\)), phosphate (PO\(_4\)^{3-}\), and carbonate (CO\(_3\)^{2-}\). Also, biological apatite has numerous nano-scaled defect structures. The presence of bone minerals and defect structures in biological apatite are thought to facilitate in vivo reactions, such as bone remodeling of living bone.

The major substituent in biological apatite is the carbonate ion (CO\(_3\)^{2-}\), which is generally present in bone at a rate of 5-8 mass%. Carbonate ions are typically substituted at two sites in the apatite structure. These are A- and B-type carbonate substitutions, which are substitutions of the hydroxyl and phosphate ions, respectively. The occurrence of B-type substitution alters various physical properties. Meanwhile, the presence of B-type substitution decreases the crystallinity and increases the solubility in vitro and in vivo. The major cations that are substituted in biological apatite are K\(^+\), Na\(^+\), and Mg\(^{2+}\). The Na\(^+\) in biological apatite is thought to be involved in cell adhesion and, bone metabolism, and resorption processes. The Mg\(^{2+}\) acts as a mineral in bone remodeling, and affects the nucleation and crystal growth of HAp. The K\(^+\) plays an important role in the regulation of biochemical processes and nucleation process of apatite minerals. The major anions that are substituted in biological apatite are F\(^-\) and Cl\(^-\). The F\(^-\) suppresses dental caries, and promotes cell proliferation, and differentiation. Cl\(^-\) is expressed on the bone surface under an acidic environment, which activates osteoclasts in the bone resorption process.

Considering the importance of these trace ions in biological processes, many studies have focused on substituted HAp.

We have previously synthesized bone mineral containing apatite (bone HAp) by adding the trace ions found in biological bone to HAp. The prepared bone HAp contains defective structures. Porous ceramics fabricated from bone HAp have excellent osteogenic and bioreabsorbable properties; however, they have lower strength compared to conventional HAp ceramics. In this study, we refined the raw material powder to fabricate bone HAp with the ultimate goal of improving the strength of the porous ceramics. Therefore, we investigated the effect of ball milling conditions on the sintering properties of the raw material powder.

The raw material powder for synthetic bone HAp was according to the previous report. Previous
studies have used 0.5 hours milling; however, we studied longer ball milling time of up to 4 hours. The resulting powders were uniaxially pressure molded and then sintered under a carbon dioxide gas atmosphere with steam at a specified temperature for 5 hours to produce dense ceramics. The obtained powders and ceramics were evaluated by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), and scanning electron microscopy (SEM).

MATERIALS AND METHOD

Preparation of Bone HAp Powders

Bone HAp powders were prepared by a traditional wet process as shown in Table 1. Calcium hydroxide (Ca(OH)$_2$), magnesium chloride hexahydrate (MgCl$_2$·6H$_2$O), sodium chloride (NaCl), potassium chloride (KCl), phosphoric acid (H$_3$PO$_4$), ammonium fluoride (NH$_4$F), and ammonium carbonate ((NH$_4$)$_2$CO$_3$) were used as starting chemical precursors for Ca$^{2+}$, PO$_4^{3-}$, Na$^+$, K$^+$, Mg$^{2+}$, Cl$^-$, F, and CO$_3^{2-}$, respectively. We assume that the Na$^+$ and K$^+$ ions are preferentially located in Ca(1) sites (Screw Ca$^{18}$). Mg$^{2+}$ ions are preferentially located in Ca(2) site$^{19}$. On the other hand, in case of simultaneous substitution of Mg$^{2+}$ and CO$_3^{2-}$ ions, Mg$^{2+}$ ions are preferentially located in Ca(1) site$^{20}$. F$^-$ and Cl$^-$ ions are located in OH$^-$ ions$^{21}$.

For the preparation of an aqueous suspension, an appropriate amount Ca(OH)$_2$, MgCl$_2$·6H$_2$O, NaCl, KCl and (NH$_4$)$_2$CO$_3$ was mixed in pure water with vigorous stirring at a rate of 200 rpm. An aqueous solution containing H$_3$PO$_4$ and NH$_4$F was then added dropwise to the suspension over a period of 3 hours. The pH of the suspension was maintained at 8.7 by dropwise addition of hydrochloric acid (HCl) solution for 3 hours. The precipitates, the precipitated suspension was then aged at 37°C for 3 days. The resulting precipitates were washed and filtered repeatedly with pure water, and then dried in an oven at 110°C for 2 days. The resulting dried cakes were ground to fine powders and calcined at 800°C with a heating rate of 5 °C·min$^{-1}$ for 1 hour under a CO$_2$ gas including steam atmosphere.

The powders obtained above were crushed by ball milling for 0.5, 1, 2, and 4 hours. To a zirconia pot, 10 g of powder, 40 cm$^3$ of pure water, and 180 g of zirconia beads with a diameter of 2 mm were added, and then ball-milled at a rotation rate of 300 rpm. The powders were then freeze-dried for 1 day and each powder was denoted as “Bone HAp(x-y),” where x is the milling time in hours.

Fabrication of Bone HAp Ceramics

Crushed bone HAp powders were uniaxially compressed at 100 MPa to form disk-shaped compacts (diameter and thickness of 10 mm and 2–3 mm, respectively) and cylindrical-shaped (diameter and height of 4.2 mm and 7–8 mm, respectively) compacts. The bone HAp ceramics were fabricated by heating the compacts to 1200, 1250, 1300, and 1350°C at a rate of 5°C·min$^{-1}$ for 5 h under a CO$_2$ gas with steam atmosphere. Each ceramic was denoted as “Bone HAp(x-y),” where x and y were the milling time (hours) and heating temperature (°C), respectively.

Characterization of powders and the prepared ceramics

Crystalline phases of the powders and ceramics were determined by powder XRD using a MiniFlex from Rigaku Co. (Tokyo, Japan) with a Cu-K$_{α}$ radiation source at 30 kV and 15 mA. XRD data were collected with 2$θ$ range, scan rate, and sampling width of 10°–50°, 2° per minute, and 0.02°, respectively. The lattice constants of the crushed ceramics were determined by refinement of XRD data collected under the following conditions: 20 range of 10 to 50°; scan rate of 0.1° per minute and sampling width of 0.02° (40 kV, 40 mA; Ultima IV, Rigaku Co.). Integrated power XRD software PDFX from Rigaku, Co. was used for refinement of lattice parameter as per the manufacturer’s instructions.

The IR spectrum of specimens were obtained using an FT-IR spectrophotometer Prestige-21 from Shimadzu Co. (Kyoto, Japan) in the region of 400–4000 cm$^{-1}$ with a spectral resolution of 4 cm$^{-1}$. The samples were prepared as potassium bromide pellets.

Ultra-structures of the powder particles were observed by high-resolution transmission electron microscopy (HR-TEM) using a JEM-2100F from JEOL Ltd. (Tokyo, Japan) at 200 kV. The samples were prepared by dispersing the particles in ethanol and then collecting the particles on carbon-coated copper grids.

Particle morphology of the bone HAp powders and microstructure of the bone HAp ceramics were observed by SEM using a JSM6390LA from JEOL Ltd. (Tokyo, Japan) at 10 kV. The SEM samples were prepared by fixing the samples on pieces of double-sided carbon tape before deposition with platinum particles in a vacuum.

| TABLE 1 Preparation for bone HAp powders. | Concentration | Volume |
|-----------------------------------------|---------------|--------|
| Ca(OH)$_2$                             | 0.535         |        |
| MgCl$_2$·6H$_2$O                        | 0.0107        |        |
| NaCl                                   | 0.0226        | 1.0    |
| KCl                                    | 0.0023        |        |
| (NH$_4$)$_2$CO$_3$                      | 0.0795        |        |
| H$_3$PO$_4$                             | 0.333         |        |
| NH$_4$F                                 | 0.00263       | 1.0    |
The particle size distribution of the raw material powders was measured using a laser diffraction/scattering particle size analyzer LA-300 from Horiba Ltd. (Kyoto, Japan). For the measurement, pure water was used as a solvent and the refractive index was set to 1.60.

The Ca, P, and cation (Na, K, Mg) contents in the ceramics were determined using an inductively coupled plasma atomic emission spectrometer (ICP-AES: SPS7800) from Hitachi High-Tech Science Co. (Tokyo, Japan). First, the ceramics were ground in a mortar and 0.1 g of the resulting powder was dissolved in 1 cm$^3$ of nitric acid and then scale-up to 100 cm$^3$. The 1000 ppm solution was diluted to the desired concentration, and the content of various elements in the Bone HAp ceramics was determined by the calibration curve method.

The compressive strength of a prepared ceramic was measured using a cylindrical-shaped ceramics method according to the “Compressive strength test method for fine ceramics” (JISR1608).

The relative density of a resulting ceramic was calculated by dividing the measured bulk density by the theoretical density of HAp (3.16 g·cm$^{-3}$).

**RESULTS AND DISCUSSION**

**Characterization of bone HAp powders**

The XRD patterns of the bone HAp powders prepared at different ball milling times are shown Figure 1(A). Regardless of the milling time, each powder indicated a crystalline phase of single phase HAp.

The FT-IR spectra of the bone HAp powders are shown in Figure 1(B). PO$_4$ and OH absorptions were observed for each sample. For the bone HAp powders, absorption bands assigned to P-O stretching vibrations were detected at 960, 1030, and 1100 cm$^{-1}$. The bands assigned to O-P-O bending were found at 570 and 600 cm$^{-1}$.

The CO$_3^{2-}$ groups in the bone HAp powder were most likely formed via B-type carbonate substitution, in which CO$_3^{2-}$ is substituted for PO$_4^{3-}$.

Representative TEM micrographs of the bone HAp powders, together with the SAED images: (a) Bone HAp(0.5), (b) Bone HAp(1), (c) Bone HAp(2) and (d) Bone HAp(4).
powders are shown in Figure 2. The particle sizes were 20–50 nm in the samples regardless of the milling time. There were no observable differences in morphology. In addition, the selected area electron diffraction image showed concentric patterns; thus, all samples were likely polycrystalline.

Representative SEM micrographs of the prepared bone HAp powders are shown in Figure 3(A). The agglomeration of all powders was confirmed. In particular, Bone HAp(1) powder that was milled for one hour showed smaller and fewer aggregates than the other powders. Meanwhile, Bone HAp(2) and (4) powders that were milled longer showed larger aggregates. This suggests that long milling times may cause reaggregation of powders.

The particle size distributions of the prepared bone HAp powders are shown in Figure 3(B). The particle size of Bone HAp(1) was smaller and more uniform than the other powders. The particle size distributions of Bone HAp(2) and (4) powders showed two peaks. The wet ball-milling with zirconia beads can mill the particles down to nano-size, but the closer the particle size is to 1 μm, the more likely it is to reaggregate in water. From the SEM image and particle size distribution measurement results, the particle size of Bone HAp(1) was about 1 μm. Therefore, it is considered that once the particles size of Bone HAp(2) and Bone HAp(4) powders was reduced down to the limit size, and that Bone HAp(2) and Bone HAp(4) powders re-aggregated during further ball-milling process. The median diameters were 6.74, 2.21, and 5.94 μm for Bone HAp(0.5), (1), and (2), respectively. These values were roughly consistent with the SEM results.

Material properties of the prepared bone HAp ceramics

Ceramics were fabricated using the Bone HAp(1) powder, which had the smallest particle size. Its material properties were examined. The heating condition used was 1200°C for 5 h as in a previous report\(^\text{17}\). The Bone HAp(0.5) was also used for comparison.

The XRD patterns of the fabricated bone HAp ceramics are shown in Figure 4(A). Both bone HAp ceramics remained as single phase of HAp even after heating. The lattice constant of Bone HAp(1-1200) was similar to Bone HAp(0.5-1200) as reported in previous studies\(^\text{10}\). The \(a\)-axes of Bone HAp(1-1200) and Bone HAp(0.5-1200) were of 0.94207(7) and 0.93475(12) nm, respectively. And, the \(c\)-axes of Bone HAp(1-1200) and Bone HAp(0.5-1200) were of 0.68835(10) and 0.68873(14) nm, respectively. Also, both ceramics provided values that were close to the international centre for diffraction data (ICDD) card values (HAp, #09-432); however, slight increases in the a-axis were observed.

The FT-IR spectra of the fabricated bone HAp ceramics showed absorptions that were attributable to PO\(_4\)\(^{2-}\) and OH\(^-\) as shown in Figure 4(B). Similar results were found for the bone HAp powders as described previously (Figure 2). In addition, absorptions attributed to CO\(_3\)\(^{2-}\) were observed at 883, 1465, and 1542 cm\(^{-1}\), indicating that the ceramics were a carbonate-containing apatite. Similarly, the CO\(_3\)\(^{2-}\) in the bone HAp ceramics were derived predominantly through A-type carbonate substitution, in which OH\(^-\) was substituted by CO\(_3\)\(^{2-}\). The CO\(_3\)\(^{2-}\) moved from the phosphate group position to the hydroxyl group position due to the high temperature of 1000°C or higher.

The quantitative results for Ca, P, Na, K, and Mg elements in bone HAp ceramics fabricated by firing at 1200°C for 5 hours are summarized in Table 2. The two studied ceramics showed no significant difference in their chemical compositions.

SEM images of the surfaces on bone HAp ceramics heated at 1200°C for 5 hours are shown in Figure 5. The relative densities of the two bone HAp ceramics were both 94%. Using the SEM images of these ceramics, the average grain size was calculated

FIGURE 3 (A) SEM micrographs of the bone HAp powders: (a) Bone HAp(0.5), (b) Bone HAp(1), (c) Bone HAp(2) and (d) Bone HAp(4); (B) Particle size distribution of the ball-milled bone HAp powders.
from 200 grains by the intercept method. The grain sizes of the ceramics were 1.74 and 1.56 μm in Bone HAp(0.5-1200) and Bone HAp(1-1200), respectively. The compressive strengths were 55.7 and 63.1MPa, respectively. The improvements in compressive strength were most likely due to the smaller average grain size of the ceramics, which was caused by the finer and more uniform raw material powders used.

Material properties of bone HAp ceramics fabricated by firing at 1200°C–1350°C for 5 hours

To examine the sintering ability, ceramics with different sintering temperatures were fabricated. The results for the relative density, grain size, and compressive strength of the prepared ceramics are shown in Figure 6. The average grain sizes (Figure 6(B)) of all the ceramics increased as the firing temperature was increased. In particular, the grain size of Bone HAp(1) was slightly smaller at all firing temperatures. This was likely due to the difference in the sintering properties of the ceramics caused by raw material powder. Figure 6(C) shows the compressive strength of the resulting Bone HAp ceramics. The compressive strength of Bone HAp(1) was higher than Bone HAp(0.5) ceramics at all firing temperatures. Although there was no statistically significant difference in the compressive strength of Bone HAp ceramics, Bone HAp(1) showed a trend toward higher values. Since the relative densities of Bone HAp(1) was the similar to that of Bone

|                  | Ca mass% | P mass% | Na mass% | K mass% | Mg mass% | Ca/P molar ratio | (Ca+minerals)/P molar ratio |
|------------------|----------|---------|----------|---------|----------|----------------|---------------------------|
| Bone HAp(0.5-1200) | 41.03    | 18.23   | 0.40     | 0.49    | 0.16     | 1.69           | 1.84                      |
| Bone HAp(1-1200)  | 42.02    | 18.82   | 0.38     | 0.42    | 0.16     | 1.68           | 1.82                      |
HAp(0.5) ceramics; thus, it was considered that the microstructure of Bone HAp(1) ceramics contributed to the improvement of its compressive strength. The Hall-Petch equation has been used to find the relationship between the yield strength and average grain size of a material. Based on the yield strength, slippage, and yielding of particles occurs simultaneously; thus, smaller average grain sizes will suppress slippage. As a result, the material strength will be improved. When we compared the average grain size and compressive strength of ceramics fired at 1200°C for 5 h, Bone HAp(0.5) had an average grain size and compressive strength of 1.74 μm and 55.7 MPa, respectively. For Bone HAp(1), the values were 1.56 μm and 63.1 MPa, respectively. These clearly suggest that the compressive strength was improved due to the smaller average grain size of a ceramic, which was caused by a finer and more uniform raw material powder.

CONCLUSION

In this study, we examined the effect of ball milling time on the sintering ability of the prepared bone HAp powder. The bone HAp powders were found to be single phase apatite regardless of the milling time. The bone HAp powders were B-type carbonate-containing apatite based on FT-IR results. The carbonate ions were substituted with the PO$_{4}^{3-}$ ions in the case of wet HAp synthesis. The powder milled by ball milling for 1 hour showed the smallest and most uniform distribution based on the SEM and particle size distribution experiments. For the ceramics heated at 1200°C for 5 h, the Bone HAp(1) had smaller average grain size and higher compressive strength than Bone HAp(0.5). This was due to the use of Bone HAp(1) powder with finer and more uniform particle size, which reduced the average grain size of the ceramics and suppressed particle slippage; thus, improving the compressive strength of the resulting ceramic.

The compressive strengths of the bone HAp ceramics were affected by the refinement and homogenization of the bone HAp powder used. With the easily-sinterable Bone HAp(1) powder as a raw material in the development of porous ceramics, we expect the realization of a new artificial bone material with sufficient strength and excellent osteogenic ability.

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