Characterization of Hydroxyapatite Derived from Bovine Bone

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ABSTRACT—*In the present study, hydroxyapatite (HAp) was synthesized from biosources bovine bone in a cost effective and ecofriendly way. Bovine bone were converted to hydroxyapatite (HAP) by a heat treatment method at different temperatures. The final product were characterized by X-ray diffraction, Scanning Electron Microscopy, Energy Dispersive X-ray spectroscopy (EDX) and FT-IR. The phase, purity and crystallinity of different calcined HAp powder were analysed. It confirms that material prepare from biosources cow bone is hydroxyapatite indeed. The natural HAp obtained by calcining at 850 °C shows the desired quality. In addition, SEM results revealed the formation of microstructured HAp 0.4 μm at 700 °C and crystal agglomeration was observed with an increase in calcination temperature. Calcium and Phosphorus contents were 20 % and 11.4%, respectively, which corresponded to the Ca/P molar ratio of nonstoichiometric hydroxyapatite. The specific surfaces area of products were measured by BET method. The volume of micropores was determined.*

Keywords— Hydroxy apatite, Cowbone, heat treatment, particle size

1. INTRODUCTION

Bones consist of organic (30%) and inorganic compounds (70%). Mineral parts of bones provide their stiffness and proper mechanical properties. The model compound corresponding to a mineral phase of bones is a nonstoichiometric hydroxyapatite (HAp), i.e. HAp whose molar ratio of calcium to phosphorus is different from 1.67. Biological apatites are the components of bones and also pathological tissue (urolith, tooth scale and mineralized soft tissue) [1-3]. Those apatites are nonstoichiometric; in enamel and dentine the Ca/P molar ratio exceeds 1.67. Due to its chemical and structural similarity to bone minerals, hydroxyapatite is a promising candidate for bone substitutes. Hydroxyapatite is not only a biocompatible, osteoconductive, non-toxic, non inflammatory and non immunogenic agent, but also bioactive, i.e. it has got the ability to form a direct chemical bond with living tissues [4-5]. There are a few methods of extracting hydroxyapatite from animal bones: thermal decomposition, sub-critical water process and alkaline hydrolysis [5-6].

Hydroxyapatite (HAp) [Ca10(PO4)6(OH)2], is considered to play a vital role in various fields including the replacement of bone tissue reconstruction of skull defects, tissue engineering, artificial bone synthesis, biosensor, removal of heavy metals, and as drug carrier [10-14]. Good results have been obtained through several synthetic methods but these methods are rather complicated and necessitate a biologically hazardous process involving the evolution of ammonia, time consuming processes, and gelation/aging, drying and sintering also require precisely controlled reaction conditions [15]. The advantages of HAp from natural sources is inexpensive and uncomplicated. The thermal calcination method is commonly used for the isolation of natural HAp. Micro structural HAp has already been obtained from fish bone by thermal treatment, thermal decomposition, alkaline hydrothermal, sub critical water process from bovine bone, teeth and bones of pig, extracted human teeth and cuttle fish [16-19]. Although much has been learned about HAp isolation from natural sources, the most important parameter, exact isolation temperature, remains poorly understood. In general, it is known that Indonesian cow bone consists of HAP (57%) and gelatinous organic matter (33%). To prevent pollution by this organic matter, cow bone was calcinated. The waste of cow bone has recently become a serious issue in coastal areas of Indonesia; one of the simplest ways to decrease pollution is the selective isolation of HAp from this waste. The research was conducted with the aim to prepare and characterize the hydroxyapatite from cow bones for bone substitution. The main objectives are to prepare hydroxyapatite(HA) from cow bone, to characterize and compared hydroxyapatite from cow bone with synthetic hydroxyapatite.

2. MATERILAS AND METHODS

2.1. Sample Preparation

In the sample preparation steps, Bovine bone are soaked in the acetone for an hour to removed collagen, fats and other impurities. Then it washed with distill water and let it dried. The bones were placed in an open silica crucible and heated in an electric furnace under ambient conditions, at different temperatures ranging from 200 °C to 1000 °C with 5 hours holding time. Then the bones were ground into 200 μm particle size.

2.2. Characterization

Mass loss pattern during heating and energy loss or absorbed measurement were detected by Thermo gravimetric analysis (TGA) and Differential scanning calorimetry (DSC). We used Pyris 7 TGA/DSC analyzer, Perkin Elmer Inc., U.S., with scan range from 50 to 1000 °C, at constant heating rate of 10 °C/ min, with continuous nitrogen flow. The stretching frequencies (vibrational origin) of samples were examined by Fourier Transform Infrared Spectroscopy, Perkin Elmer (U.S.) and spectrum GX spectrometer within the range of 450 to 4000 cm^{-1}. The phase and crystallinity of HAp were evaluated using X-ray diffractometer (Phillips, PW-3710) Co-Kα radiation with wave length 1.78896 Å and over a range 2θ from 20° to 80° angle, step size 0.02, scan speed 4°/min with 40 kV voltage and 30 mA current. The X-RD pattern were compared with literature profile JCPDS 09-0342/1996 to identify the phase. Morphology and chemical elements of HAp crystals was obtained by field emission scanning electron microscopy (FE-SEM JSM-6700F, JEOL, Japan) equipped with an *in situ* energy dispersive X-ray (EDX) spectrometer.

3. RESULTS AND DISCUSSION

3.1 Thermal Analysis of Bovine Bone

The removal of the organic portion from bovine boneconfirmed by TGA and DSC analysis and the results are shown in **Figure** 1. In the TGA and DSC curves, two inflection points were observed in the bovine boneat 100.5 °C and 350°C which corresponds to removal of the water and organic matter. No significant weight loss was observed between 700 °C and 1000 °C, indicating the complete removal of organic materials such as collagen, chondroitin sulfate, keratin sulfate, and lipids below 700 °C.

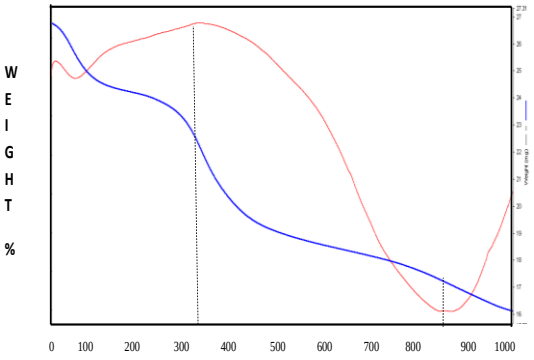


Figure 1: TGA and DSC curves of cow bone

The removal of the organic portion after calcining was observed at different temperatures with changes in the color of the bone (**Table 1**) and **Figure 2**. The color of the raw bovinebone was observed as light yellow, which consequently changed into black, tarnish and off-white when subjected to calcination at 200°C ,300 °C, 350 °C 400 °C, 500 °C and 600 °C temperatures, respectively. The color of the Cow bone turned white with further increase in the temperature. The different colors observed below 700 °C revealed the association of the organic matrix within the bone. It is evident from the results that different degrees of removal of organic portion were observed at varying temperatures, with 700 °C being the lowest optimal temperature to produce HAp with almost no organic substances associated. The isolation yield of HAp at different temperature 700 °C, 900 °C and 1000 °C were 61.52%%, 57.23% and 56.86% respectively.

Table 1.Residues and color of calcined Bovine Bone

No	Temp. (°C)	Initial Weight (g)	Loss of Weight (g)	After (g)	% Residu	Color
1.	Raw Cow Bone					Yellow
2.	200	100	8.53	91.47	91.47%	Yellow
3.	300	100	19.19	80.81	79.42%	Black
4.	350	100	34.25	65.75	65.75%	Black
5.	400	100	34.47	65.53	65.53%	Black
6.	500	100	35.09	64.91	64.91%	Tamish
7.	600	100	35.35	64.65	64.65%	Grey
8.	700	100	38.48	61.52	61.52%	White
9.	800	100	41.11	58.89	58.89%	White
10.	850	100	42.37	57.63	57.63%	White
11.	900	100	42.77	57.23	57.23%	White
12.	1000	100	43. 14	56.86	56.86%	White

3.2 FTIR spectroscopy

FT-IR analysis, in the transmittance mode, shows the presence of carbonate group at around 1410-1450 cm^{-1} and 873 cm^{-1}, hydroxide group at around 3500-3200 cm^{-1}. For phosphate group at 1030-1090 cm^{-1} and 1950-2200 cm^{-1}, 962 cm^{-1} and 560 cm^{-1}. Based on the graph, the value for (a) 3420-3570 cm^{-1} and 1640cm^{-1}(b)1030-1090 cm^{-1}, 960cm^{-1}, 560-600 $cm^{-1}$and 460 cm^{-1}(c) 873 cm^{-1} and (d) 1410-1450 cm^{-1} and 873 cm^{-1}. From the result, it is known that (a) producing hydroxide group, (b) phosphate ion while (c) and (d) produced carbonate ion.

On the **Figure 2**. Phosphate(PO_4^{3-}) asymmetric stretching vibration calcination temperature of350°C (A), 400°C(B), 500°C(D) and600°C(D) was detected only at wave number 1040 cm^{-1}. Asymmetric bending vibration for 350°C, 400°C, 500°C and 600°C calcination results shown in wave number 550 cm^{-1} to 600 cm^{-1}. For symmetric stretching vibrations result calcinations temperature of 300°Cto 600°C appear at wave number 960 cm^{-1} but also seen a wave number 880 cm^{-1}. Symmetry bending vibration results calcinations temperature of 300°C to 600°C was detected at wave number 436 cm^{-1}, but in the second the result of calcinations temperature of 300°C to 600°C detected the existence of CO_3^{2-} is the wave number 1460-1380 cm^{-1} and wave number 873 cm^{-1}.

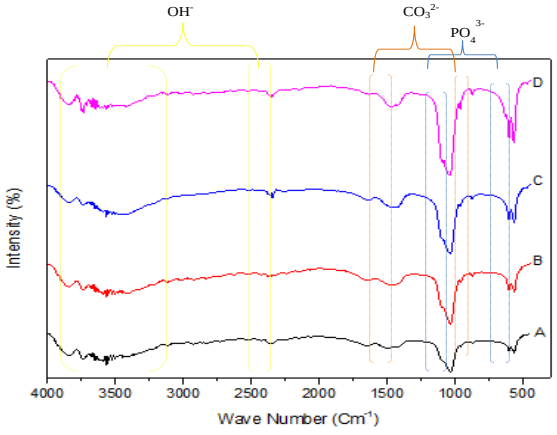


Figure 2: FTIR results of cow bones after calcined at 350°C (A) , 400^0 C (B), 500^0 C (C) and 600°C (D)

On **Figure 3:**. Phosphate (PO_4^{3-}) asymmetric stretching vibration calcinations temperature of 700°C to1000°C was detected at wave number 1030 cm^{-1} to 1090 cm^{-1}.

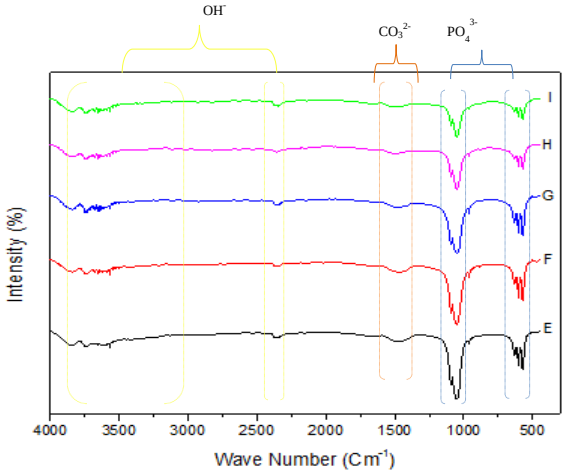


Figure 3:. FTIR results of cow bones after calcined at 700 °C, 800 °C, 850°C, 900 °C and 1000 °C

Asymmetric bending vibration for both calcinations results shown in wave number 580 cm^{-1} to 640 cm^{-1}. For symmetric stretching vibrations result with calcinations temperature of 600°C to1000°C appear at wave number 960 cm^{-1}. Symmetry bending vibration results with calcinations temperature of 600°Cto1000°C was detected at wave number 436 cm^{-1}, but in the second the result of with calcinations temperature of 600°C to1000°C was also detected CO_3^{2-} is the wave number 1460-1380 cm^{-1} and wave number 874 cm^{-1} with the amount absorbed smaller percentage.At lower temperatures (350 °C to 600 °C), the peak corresponding to the phosphate (PO_4^{3-}) group at 1090 cm^{-1} was not observed and it appeared only at temperatures above 600 °C. This may be due to the removal of all the organic material from the raw bovine bone and formation of HAp crystals. The FT-IR spectrum of bovine bone calcined between temperatures of 700 °C to 1000 °C revealed the only characteristic peak of HAp, which is consistent with some previous reports [20-21] .

A large number of bands in the spectra (601, 631, 873, 962, 962, 1027, 1088, 1413, 1454, 2034, 2157 cm-1 and a broad band observed between 3300–3600 cm-1) matched the bands in the HAp reference spectrum and are in close agreement [22]

FT-IR analysis, in the transmittance mode, shows the presence of carbonate group at around 1410-1450 cm^{-1} and 873 cm^{-1}, hydroxide group at around 3200-3500 cm^{-1}. For phosphate group at 1030-1090 cm^{-1} and 1950-2200 cm^{-1}, 962 cm^{-1} and 560 cm^{-1}. At lower temperatures (350 °C, 400 °C and 500 °C), the peak corresponding to the phosphate (PO_4^{3-}) group at 1088 cm^{-1} was not observed and it appeared only at temperatures above 600 °C.

This may be due to the removal of all the organic material from the raw bovine bone and formation of HAp crystals.

3.3 . X-Ray Diffraction Analysis

The phase and purity of derived HAp crystals were confirmed with XRD analysis. **Figure 4** shows the XRD pattern of raw bovine bone and heat treated bone at different temperatures. The XRD pattern show that the HAp stability in the bone matrix was not disrupted when calcined in air up to 1000 °C, as the chemical structure of HAp has not been affected and no other peak was obtained apart from HAp. The crystalline composition of calcined bovine bone was found to be similar to that of HAp (JCPDS-09-0432/1996) when calcined between 700–1000 °C. It is well known that as the temperature increases the intensity of the peak increases with a decrease in the peak width. The intensity of the raw bovine bone was found to be dispersed by x-ray radiation with a lowered intensity and wider peak. This may be due to the presence of extracellular matrix and fibrous proteins. When subjected to calcination at higher temperatures, the subsequent peaks were highly intense and sharp, indicating the removal of organic portion [21].

These diffraction patterns show a gradual increase in the degree of sharpness of peaks with increasing heat treatment temperature. These results confirm the previous discussion regarding the effect of the heat treatment temperature on the crystal size of hydroxyapatite. From the pattern, it can be seen that the sample with the temperature of 1000°C has the highest intensity compared to the other temperature. The temperature of 1100°C was detected has the minor impurity elements and the most suitable to produce the best HA.

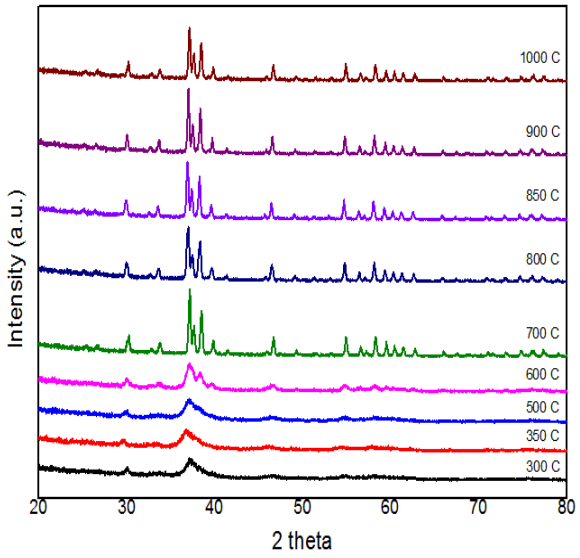


Figure 4: the XRD pattern of raw *Cow* bone and heat treated bovine bone at different temperatures

Table 2 shows the crystallographic parameter obtained from XRD spectra. The obtained d-spacing lines, 2θ angle, relative intensity at the different temperatures, have been compared with standard HAp (JCPDS-09-0432/1996) and the error was estimated at every plane. From these results, it is evident that HAp derived at different temperatures is very close to the standard HAp in purity and stability.

The relative intensity of calcined bone was found to be closest to standard HAp at 800 °C. The 2θ angles varied a little in comparison to standard HAp, which might be due to the trace removal of OH radicals. Dehydroxylation of the HAp phase would cause a small degree of peak shifting in the XRD trace.

Table 2: Crystallite size, Density, Cell Unit, Crystallite System of obtained HAp

No	Temp °C	Crystallite size (A^0)	Density (g/cm3)	Cell Unit	Crystallite System
1	700	257.7	3.16	a=b=9.4092 c=6.87539	Hexagonal $\alpha=\beta=90^0$: $\gamma=120^0$
2	800	223.3	3.17	a=b=9.3975 c=6.86525	Hexagonal $\alpha=\beta=90^0$: $\gamma=120^0$
3	900	581.5A^0	3.17	a=b=9.4047 c=6.8705	Hexagonal $\alpha=\beta=90^0$: $\gamma=120^0$
4	1000	254.2	3.17	a=b=9.4064 c=6.8711	Hexagonal $\alpha=\beta=90^0$: $\gamma=120^0$

In the present work, it was found that 2θ positions of the bone samples calcined at 700 °C and 1000 °C shifted by total error of 0.056 and 0.031, respectively, thus indicating that the HAp lattice has contracted due to loss of OH radicals.

It should be noted that although the decomposition of HAp phases was not detected in samples calcined at 1000 °C, this can be observed by simply comparing the XRD peak's position which correspond to the higher intensities planes, (0 0 2), (2 1 1), (1 1 2), (3 0 0), (2 0 2) (3 1 0) and (2 1 3) of calcined bovine bone.

Then to obtain a level of perfection information Hap crystal formation, was calculated (degree of crystallinity) using the equation:

$$Xc\ (\%) \approx 1-(V_{112/300} / I_{300})$$

Where

$V_{112/300}$ = Valley intensity between peaks 112 and 300

I_{300} = Peak intensity 300

Xc value is a fraction of crystalline phases that exist in the sample where:

Xc >70%	usually considered to be a high degree of crystal
30% >Xc >70%	Medium degree of crystal
Xc <30%	low degree of crystal

By using the formula above then degrees of crystalline of Hap could be calculated and tabulated at Table 3 below

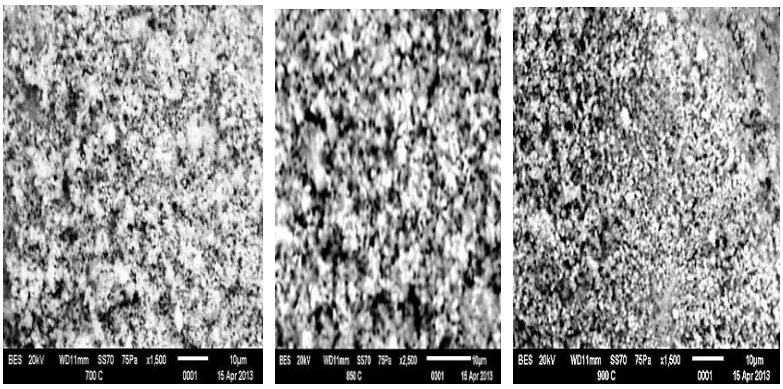
Table 3 : Degree of crystallinity for HAp bovine bone with different calcination temperature

No	Temperature ^0C	Degree ofcrystallinity	Degree of crystallinity
1.	1000	89.8 %	High
2.	900	89.44 %	High
3.	800	87.28 %	High
4.	700	88.46 %	High
5.	600	38.35 %	Medium
6.	500	Not Separated Peak Between 112 And300	Amorf

XRD analyzes were performed on Hap powder from cow bones are calcined with temperature variations between 200^0C-1000^0C resulting from this research is the HAp phase. It's the same result as shown in the sample from Aldrich HA powders. It is known from XRD peaks of phase $Ca_{10}(PO_4)_6(OH)_2$ are referenced from ICDDNo.09-0432. XRD patterns were generated in this study in accordance with the XRD pattern generated from other HAp synthesis studies.

3.4 Scanning Electron Microscope (SEM)Analysis

The surface morphology and crystal size of the derived HAp were studied under SEM. **Figures 6** (a), (b), and (c) show SEM pictures of cow bone derived HAp at 700 °C, 850 °C and 900 °C, respectively



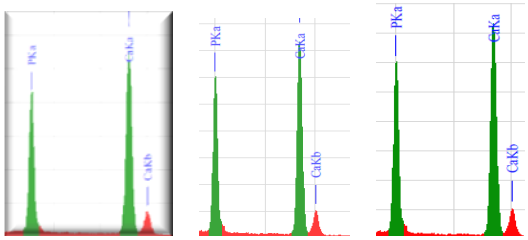
(a) (b) (c)

Figures 5: (a), (b), and (c) show SEM pictures of cow bone derived HAp at 700 °C, 850 °C and 900 °C

Microcrystal of HAp in the natural bone is very small, with a crystalline size of 5–10 nm, 10–15 nm wide and more than a few micrometers long. In Figure 6(b), formation of microparticles was clearly evident in the derived HAp at 700 °C with crystal sizes 416 nm. Whereas, in Figure 6(c) and (d), HAp microstructures were observed with increase in temperature from 850 °C to900 °C. The crystallite size of derived HAp at higher temperatures 850 °C and 900 °C is 600 nm and 833 nm respectively. It was conjectured from the surface morphology that the crystallite size increases with respect to the temperature. The formation of these microstructures of derived HAp in the thermal process can be attributed to the tendency of particles to crystallize and agglomerate at high temperature.

3.5 Energy Dispersive X-ray Analysis

EDX is an analytical technique used for elemental analysis or chemical composition of a sample. **Figure 6** (A), (B) and (C) represent the EDX data for derived HAp at 700 °C, 850 °C and 900 °C, respectively. Based on the EDX signatures, the Ca/P weight ratio for derived HAp was calculated and was found to be 1.810, 1.696 and 1.911at 700 °C, 850 °C and 900 °C, respectively; the resultant values are consistent with previous values reported elsewhere. As the Ca/P weight ratio of the derived HAp at different temperatures did not show any considerable difference, it can be inferred that Ca/P weight ratio is independent of calcination temperature.The ratioofCa/PHApcalcinedcattlebonesat a temperature of700°C, 850°Cand900°Cshowed noappreciable differencesignifican



(a) (b) (c)

Figure 6: .EDX analysis of bovine bone calcine at (**a**) 700 °C, (**b**) 850 °C and (**c**) 900 °C.

It can be conclude that the temperature change does not significantly affect the value of Ca/P in HAp calcined cowbones.

3.6 The specific surfaces area of the derived HAp by BET method

The specific surfaces of the powders obtainedwere characterized by BET method. The surface andvolume of pores were also analysed. The results arepresented in **Table 4**.

Table 4:. Surface area of the derived HAp

Parameter	Calcinated at700 °C	Calcinated at850 °C	Calcinated at900 °C
BET surface area (m2/g)	3.76	1.912	1.775
Total (cm3/g)	0.001918	0.000975	0.000904
Average pore diameter(nm)	98.89	96.9	91.69

The BET result showed that the surface area of the derived HAp at 700 °C, and 850 °C was larger than the surface of derived HAp at 900 °C. This could be caused by a carbon residue in the material obtained at 700 °C. The reduction of a specific surface and average pore diameter confirmed that the grains of powder were sintered. The average pore diameters were calculated and proved to be 98.89 nm, 96.9 and 91.69 nm for the derived HAp at 700 °C, 850 °C and 900 °C, respectively

4 CONCLUSIONS

Based on the results of this study It conclude that calcination of raw bovine bone at 700 °C to 1000 °C led to the formation of pure HAp. The crystalline nature of HAp was found to be directly proportional to the calcining temperature; the higher the temperature, the greater the particle size. When compared with the standard JCPDS-09-0432/1996 data, it confirms that the purity of derived HAp by the thermal calcinations method is good enough. In addition to this, the formation of microcrystals was observed at temperatures above 700 °C. The temperature between 700–900 °C is optimum for isolation of HAp from bovine bone with almost no organic portion, high purity, stability, crystallinity, making it appropriate for use in biomedical applications

5 ACKNOWLEDGMENT

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